THE JOURNAL OF EXPERIMENTAL MEDICINE

THE JOURNAL OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

VOLUME TWENTY-THIRD

WITH ONE HUNDRED AND FIFTEEN PLATES AND THIRTY-SEVEN FIGURES IN THE TEXT

NEW YORK
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
1916

WAVERLY PRESS
The Williams & Wilkins Company
Baltimore, U. S. A.

CONTENTS.

No. 1, January 1, 1916.

No. 2, February 1, 1916.

No. 3, March 1, 1916.

No. 4, APRIL 1, 1916.

No. 5, May 1, 1916.

No. 6, JUNE 1, 1916.

IMMUNOLOGICAL STUDIES IN PNEUMONIA.

BY RICHARD WEIL, M.D., AND JOHN C. TORREY, PH.D.

(From the First Medical Division of the Mt. Sinai Hospital, and the Department of Experimental Medicine and Experimental Pathology of the Cornell Medical School. New York.)

(Received for publication, July 20, 1915.)

The immunological processes of pneumonia have been studied within recent years by many observers, among whom may be mentioned Klemperer,[1] Römer,[2] Neufeld and Haendel,[3] Wadsworth,[4] Dochez,[5] and Chickering.[6] It has been established that a certain type of immune body, namely, the agglutinins, tends to appear in the blood shortly after recovery from the disease, and to persist therein for a variable period of time. During the course of the disease this antibody has only exceptionally been found. In addition, specific protective substances have been found in convalescent serum.

During the past winter we have approached the problem with the help of a method not previously employed in the study of this disease. Essentially, the method consists in passively sensitizing guinea pigs with serum taken from cases of pneumonia at various stages of the disease, and during convalescence. Passive sensitization of guinea pigs with human serum had previously been practised by Bruck[7] and Schloss in the study of food idiosyncrasies. The application of the method to the study of infectious diseases has been attempted in the case of tuberculosis.

[1] Klemperer, G., and Klemperer, F., *Berl. klin. Wchnschr.*, 1891, xxviii, 833.

[2] Römer, Experimentelle und klinische Grundlage für die Serumtherapie der Pneumokokkeninfection der menschlichen Cornea (Ulcus serpens), Wiesbaden, 1909.

[3] Neufeld, F., and Haendel, *Arb. a. d. k. Gsndhtsamte*, 1910, xxxiv, 166, 293.

[4] Wadsworth, A., *Am. Jour. Med. Sc.*, 1904, N. S., cxxvii, 851; *Jour. Exper. Med.*, 1912, xvi, 54.

[5] Dochez, A. R., *Jour. Exper. Med.*, 1912, xvi, 665.

[6] Chickering, H. T., *Jour. Exper. Med.*, 1914, xx, 599.

[7] Bruck, C., *Arch. f. Dermat. u. Syph.*, 1909, xcvi, 241.

One of the most serious difficulties associated with this method lies in the fact that human serum is extremely toxic to guinea pigs. After a considerable amount of preliminary investigation we finally adopted the use of inactivated serum, as recommended by Schloss[8] and others. For the sake of uniformity we have, in all except the first studies, regularly injected 4 cc. of inactivated serum into the subcutaneous tissues. After a period of time varying from two to six days, the guinea pigs have been tested to determine whether or not they presented an induced hypersusceptibility to the pneumococcus. The method of performing this test differs from that hitherto observed in this type of experiment. The routine test, as usually performed, consists in the introduction of the antigen either into the peritoneum or into the veins of the sensitized animal. In the study herein described that method was not practicable.

The antigen was prepared in the following manner. Forty-eight hour cultures on Loeffler's serum medium (ox serum) were emulsified in distilled water, using 2 to 3 cc. of water for each tube, according to its size. The emulsion was shaken and autolyzed at 37° C. for two hours, and then at 60° C. for one hour.[9] With a very few exceptions, the organism employed was of Type I. In a few of the earlier experiments a Group III type (*Pneumococcus mucosus*) was used with success. It seems probable that this anaphylactic reaction is more general for the whole pneumococcus family than has been found to be the case in agglutination and protective experiments. This autolytic extract when injected intravenously into guinea pigs is primarily toxic, but the toxicity varies with different animals to a considerable extent, so that the reaction produced in the anaphylactic experiment is unreliable and confusing. As an alternative, the induced hypersusceptibility of the guinea pigs was tested by the Dale[10] method. This method consists in removing the uterus of the sensitized animal and suspending it in a bath of Locke's fluid. The uterine preparation traces a rhythmical curve, which changes strikingly upon the addition of antigen to the bath fluid. There is an immediate, or slightly delayed, response in the nature of a sharp

[8] Schloss, O. S., *Am. Jour. Dis. Child.*, 1912, iii, 341.

[9] Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727.

[10] Dale, H. H., *Jour. Pharmacol. and Exper. Therap.*, 1912–13, iv, 167.

contraction, which may be followed either by prompt relaxation or by tetanus of the muscle. In testing the pneumococcus autolysate, it is necessary to keep in mind certain important factors. In the first place, the antigen of itself possesses in a certain degree the property of stimulating a contraction, even when added to the normal uterus. In this respect, however, it does not differ from normal sera, such as horse serum or ox serum, which have the same effect upon the normal guinea pig uterus. An essential feature of the method therefore consists in the use of the antigen in such amounts as do not, of themselves, suffice to induce contractions in the control uterus.

In the present study we have made a practice of using approximately one-quarter of that amount which just fails to induce a response in the normal uterus. As a rule, 1 cc. of extract, which has always been uniformly prepared throughout the series, is added to a bath containing 150 or 200 cc. of fluid. It is a necessary precaution to test each sample of autolysate, in order to be sure that its primary toxicity is not greater than is assumed for this amount. Occasionally it has been found necessary to diminish the amount of antigen used in the tests on account of an unexpectedly high primary toxicity. The other technical details of the method are not such as to require special discussion in this connection.

OBSERVATIONS.

Blood has been taken and tested from a series of cases which presented themselves, clinically, as pneumonias, as well as from a series of controls which were either normal individuals or cases of other forms of disease; such as typhoid, rheumatism, etc. In every case of pneumonia the sputum was cultured in order to determine the etiological organism, and in some cases blood cultures as well were made; in all, 20 cases of pneumococcus, 2 of *Streptococcus mucosus*, 2 of streptococcus, and 1 case of *Bacillus mucosus* were determined. In 9 cases the organism was not definitely determined.[11]

[11] The sputum examinations and the blood cultures were carried out for the Mt. Sinai cases by Dr. H. Celler, to whom we wish to express our indebtedness. He reported on sputa only when the plates presented practically pure cultures of the organism.

Leaving out of consideration the cases of questionable etiology, none of those cases which were bacteriologically identified as other than pneumococcus infections, with the exception of one case of *Streptococcus mucosus* infection, gave a positive response. Of the twenty cases of pneumococcus infection, there were only two which did not at some stage of their course present a positive response. Of the questionable cases, in which the bacteriological diagnosis could not be obtained, there were four in which the clinical pictures seemed, with certainty, to exclude the possibility of a pneumococcus infection. In none of these four was a positive result obtained. In

TEXT-FIG. 1. Guinea pig 583. Received 4 cc. of inactivated serum derived from a case of acute articular rheumatism. Tracing, taken on fourth day, shows no reaction to pneumococcus antigen, although there is a sharp response to ergamine.

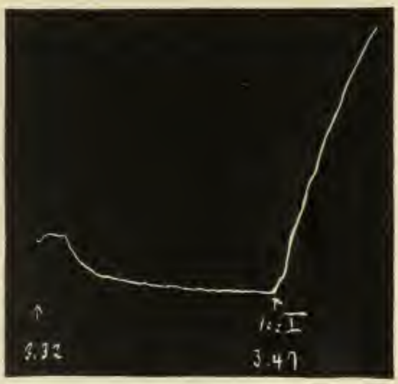
the other cases of that group it was not possible to decide, with any degree of certainty, whether or not the infection was due to pneumococcus. The results obtained in these cases were in some instances positive, and in others negative.

The time of occurrence of reaction differed from that observed by others in the study of the agglutinins. In all of the cases in which it occurred, it appeared before the temperature had reached normal. In only two of these cases was it also present during convalescence, and then in far less marked degree. The reaction was present within

two days of the onset of the disease in one case, within three and four days in certain other cases. When found, it persisted almost unexceptionally up to the time of crisis; or if the disease ended by lysis, it persisted throughout the lysis. Two of the cases ended fatally; in one of these it was found throughout the disease; in the other, which died upon the day of admission, it was not found. Those two cases in which it persisted after crisis did not appear to differ according to any clinical criteria from those in which it disappeared. In none of the control cases was the reaction induced.

The conditions which have been described may fitly be illustrated by a number of typical tracings. Text-fig. 1 shows a tracing taken from

TEXT-FIG. 2.



TEXT-FIG. 3.

TEXT-FIGS. 2 AND 3. Guinea pig 565. Received 4 cc. of inactivated serum derived from a case of acute lobar pneumonia due to pneumococcus, five days after onset. Tracings, made on fifth day, show a sharp response to antigen.

the uterus of a guinea pig which had received 4 cc. of the inactivated serum of a patient suffering from an attack of acute articular rheumatism. The temperature at the time the blood was taken was 103° F. It is evident that there is no response to the application of the pneumococcus antigen. Text-figs. 2 to 7 illustrate the course of a case of lobar pneumonia. The patient was admitted to Mt. Sinai Hospital on the fourth day of the disease. The onset had been acute and was marked by a chill. Upon admission, there were characteristic signs of consolidation of the middle and lower lobes of the right

lung. The temperature was 105.4° F., and the leucocyte count 15,200, of which 85 per cent were polynuclears. The temperature varied only slightly for four days, and on the fifth day fell by uninterrupted crisis. Recovery was uneventful from the crisis. The clinical picture was that of a lobar pneumonia of moderate severity.

Text-Fig. 4.

Text-Fig. 5.

Text-Figs. 4 and 5. Guinea pig 572. Received 4 cc. of inactivated serum derived from same case during crisis. Tracings made on third day. There is no response to antigen. Ergamine produces a sharp contraction.

Pneumococci were found in the sputum, but not in the blood. Blood was taken for the anaphylactic tests on three occasions; namely, during the height of the disease, during the crisis, and on the third day after the crisis. On each occasion the blood was subjected to the same procedure. The serum was allowed to separate from the clot,

TEXT-FIG. 6.

TEXT-FIG. 7.

TEXT-FIGS. 6 AND 7. Guinea pig 588. Received 4 cc. of inactivated serum derived from the same case three days after crisis. Tracing taken on fourth day. One horn shows a minimal response to the antigen. Both horns respond well to ergamine.

and was then centrifuged to free it of cells. It was then inactivated for one-half hour at 56° C. Of this serum, 4 cc. were injected subcutaneously into a female guinea pig weighing between 250 and 300 gm. After an interval of from three to five days the guinea pig was killed, and the uterine horns were immediately removed and suspended, according to Dale's method, in a bath containing 200 cc. of Locke's fluid. The reaction to the pneumococcus autolysate was tested, and if this was negative, ergamine was subsequently added to establish the normal reactivity of the muscle. The figures show that the blood taken during the course of the disease had effectively sensitized the uterus to the pneumococcus antigen. The blood taken during the crisis and after the crisis failed to induce this result, although the muscle evinced a normal reactivity towards ergamine.

Of interest in connection with our own results are those described by Seligmann.[12] This author reports that he attempted passive sensitization of guinea pigs with postcritical and precritical serum, but that he failed to achieve any positive result. He gives no detailed protocols of his experiments, but describes his method, which differs in practically every detail from that employed in the present study. He sensitized by means of the intraperitoneal injection of 5 to 6 cc. of the human serum. We have almost regularly found this dosage fatal for the guinea pig, and therefore inactivated the serum and gave it subcutaneously. The interval between passive sensitization and the toxic injection in Seligmann's experiments was only one day, whereas we allowed three or more days for the absorption of the human antibodies. His second injection consisted of a suspension of a pneumococcus culture, while we used the soluble autolysate. Finally, he employed the intravenous injection, whereas we tested by the Dale method. Without further analysis, it must be clear that any one of these differences in technique would suffice to account for the divergence in results.

DISCUSSION.

The question which immediately arises naturally involves the nature of the substances in the sera concerned in the production of

[12] Seligmann, E., *Ztschr. f. Immunitätsforsch.*, 1911, ix, 79.

this reaction. There is practically no doubt that they must be grouped among the immune substances, the so called antibodies. There are no other substances, so far as we know, which passively sensitize a guinea pig's uterus. Apparently they are specific in character. Whether or not they have protective value is a problem which has not been approached. The fact that their incidence in the disease differs strikingly from that of the agglutinins and the lytic antibodies, suggests the likelihood that they may be different from those substances. In fact, there is some ground for the belief that hemolysins, agglutinins, and precipitins may not be identical with anaphylactic antibodies, judging from the fact that the first group of substances does not always run parallel with the second in amount.

Such a conclusion, however, does not necessarily follow. It is possible that exactly the same substance acts as agglutinin in the test-tube and as sensitizing substance in the guinea pig, and that the apparent difference in the effects observed by the two methods is due simply to the coexistence of varying amounts of antigen. According to this view, agglutination is inhibited in the precritical serum through the presence of antigen, a factor which does not, however, inhibit passive sensitization. The postcritical serum, on the other hand, might contain less antibody, so as to impair its sensitizing value, but might at the same time be almost free from antigen, so as to permit of the demonstration of agglutinins in the test-tube.[13]

The clinical material on which this work is based was obtained chiefly from the service of Dr. Libman at the Mt. Sinai Hospital, and in part also from the service of Dr. Brill at the Mt. Sinai Hospital, and from the service of Dr. Coleman in Bellevue Hospital, for whose cooperation we desire to express our thanks. We are also indebted to Drs. Cole and Dochez of The Rockefeller Institute Hospital for pneumococcus cultures and sera.

[13] The experiments on which this theory is based are included in a series of papers by Weil, R., *Jour. Immunol.*, 1916, i (in press).

CONCLUSIONS.

The serum derived from human cases of pneumonia has been used to sensitize guinea pigs passively. As antigen an autolysate of a pneumococcus culture was employed. By means of Dale's method it has been possible to show with considerable regularity that the blood contains sensitizing antibodies during the course of pneumonia, but none after the crisis. Patients suffering from other diseases have failed to give this reaction, as have also normal individuals.

NOTE ON A SKIN REACTION IN PNEUMONIA.

By RICHARD WEIL, M.D.

(*From the First Medical Division of the Mt. Sinai Hospital, New York.*)

(Received for publication, July 20, 1915.)

The present paper describes the results observed when pneumococcus antigen, prepared in the same way as for the anaphylactic experiments described in the preceding article,[1] was injected intracutaneously into a series of pneumonia patients, and into controls. The same method has been employed by Clough,[2] who reached the conclusion that these injections had in general an irritant effect, and that no sharp differences could be shown to exist between pneumonia cases and controls. The present observations do not coincide with those of Clough, but it seems likely that the divergence in results may possibly be due to a difference in the composition of the antigen employed. A very strong pneumococcus extract possesses primarily irritative properties, and sets up reactions independently of any immunological response. In Clough's work the dried and ground residue of a twenty-four to thirty-six hour culture was extracted at 37° C. for eighteen hours. The two hour autolysate employed in the present study is very much weaker than Clough's extract. The method of injection was intracutaneous, and the site chosen was the interscapular region. The autolysate, prepared as described in the preceding paper by Torrey and Weil, was kept in the ice box, and was never used more than one week after its preparation. During this period no change could be detected in its effects when injected into the skin. Not infrequently it happens that a sediment forms in the tubes containing the autolysate; this was not disturbed, but the supernatant fluid was carefully drawn off for the tests. As a rule, the autolysate has a faint whitish, opalescent appearance. The amount injected varied from 0.1 to 0.2 cc., depending on the texture of the

[1] Weil, R., and Torrey, J. C., *Jour. Exper. Med.*, 1916, xxiii, 1.

[2] Clough, P. W., *Bull. Johns Hopkins Hosp.*, 1915, xxvi, 37.

skin. The object in giving the injection was to produce a small wheal of the skin. The injection is immediately followed by a superficial, ill defined, cutaneous blush. This is present in pneumonia cases as well as in controls. It is believed to be due to a local irritant effect, and is not considered as a true reaction. The subsequent course of events at the site of injection varies under different circumstances. The superficial erythema may fade within a few hours, and nothing further may develop. Such a course is denominated as an absence of reaction, and the case is called negative. On the other hand, within the twenty hours following the injection further changes may occur at the site of injection. A fairly well circumscribed area of erythema, with slight infiltration and elevation of the skin surrounding the point of puncture, may develop. If the infiltration is marked, a true papule results. These changes may persist for forty-eight hours or more. They are described as a reaction, and a case presenting this is called positive. No attempt has been made in the following analysis to differentiate between the grades of positive reaction, or to distinguish the significance of an indurative from a papular reaction.

During the course of the disease the skin presents no reaction to the injection. After the subsidence of the disease, a considerable percentage of the cases do present a reaction. This reaction may exceptionally be induced within twenty-four hours after the crisis, or may make its appearance only after an interval of two or three or more weeks. The normal individuals, or the diseased controls, may or may not present a reaction, depending presumably on their previous sensitization by the pneumococcus or an allied organism. Such sensitization might presumably depend upon a previous mild and unidentified attack of the disease, or upon reaction to the presence of parasitic organisms in the throat. A few cases of pneumonia have been observed in which a reaction was not only absent throughout the disease, but failed to manifest itself at any time during the after period.

DISCUSSION.

The interpretation of these results is not entirely clear, owing to the fact that the mechanism of the skin reaction has not been sufficiently elucidated. Unquestionably, there are two types of skin reaction, of which the one, exemplified by the method of Römer and Schick in the case of diphtheria toxin, depends upon the primary toxicity of the antigen. A positive response in this type indicates a deficiency in the mechanism of defense. Probably Clough's reactions fall into this category. On the other hand, the antigen may have no primary toxicity, as, for example, horse serum or tuberculin, and in this case a positive response indicates the presence of antibodies. The observations herein described were based on the use of a non-toxic concentration of the pneumococcus autolysate. In the second place, it is not known whether the reaction is mediated by the circulating or by the fixed antibodies, and in either case to what extent it is modified by the coexistence of either fixed or circulating antigen. On the basis of animal experiments, the latter factor might be assumed to play a very essential part. Finally, the human being is a very unfavorable subject for the elucidation of skin reactions, presenting, in this respect, marked individual variations. Tentatively, we should be inclined to assume that the absence of a reaction during the course of the disease was attributable not to the absence of antibody, but to the coexistence of sufficient amounts of antigen within the cells to inhibit the reaction. With the diminution of antigen, which goes on progressively from the time of crisis, antibody may become available for a reaction. If the latter is present in sufficient amounts, the cutaneous reaction to the injection of antigen is pronounced; if not, it is absent. The absence of the reaction during the disease presents an interesting analogy with the effect of an acute general miliary tuberculosis on the von Pirquet reaction.

The work of Major and Morse[3] in many respects closely parallels the observations above recorded. A moderately severe type of reaction, described as erythemo-indurative, was observed in a large number of control cases. Cases of pneumonia, bacteriologically

[3] This work has not been published. The manuscript report was kindly sent me by Dr. Hirschfelder, under whose direction it was carried on.

identified only by microscopic study of the sputum, reacted regularly shortly after the crisis, but never during the disease. The reaction usually became papular in type. Except for the fact that the pneumonia cases invariably responded shortly after the crisis, these observations agree in essential particulars with the present series. In view of the fact that Major and Morse studied only four cases subsequent to the crisis, it seems not unlikely that the regularity observed by them might have disappeared in a larger series.

From a diagnostic standpoint, the reaction has no significance. The fact that it is absent during a pneumonic process does not sufficiently establish the bacterial etiology of the disease.

AN EXPERIMENTAL STUDY OF THE EFFECT OF CHOLECYSTGASTROSTOMY ON GASTRIC ACIDITY.

By ERNEST G. GREY, M.D.

(From the Laboratory of Surgical Research of the Harvard Medical School, and the Surgical Service of the Peter Bent Brigham Hospital, Boston.)

(Received for publication, July 26, 1915.)

INTRODUCTION.

The beneficial effects of gastro-enterostomy on ulcer of the stomach and duodenum are variously explained, some authorities maintaining that they result from a more complete and rapid evacuation of the stomach, others that the effect is due mainly to chemical changes in the gastric contents which result in a reduction of the total acidity. Between these two hypotheses there is still another view which attributes the improvement to a combination of both factors. W. J. Mayo, for example, believes that gastrojejunostomy not only affords better drainage but also alters the physiology of the stomach in a favorable way. In a similar manner Hartmann and Lecene, Noetzel, Collinson, and others hold that a partial neutralization of the gastric acidity results from the reflux of jejunal contents into the stomach. Finney and Friedenwald, who have recently drawn attention to the advantages of pyloroplasty over gastro-enterostomy, report a reduction of hyperacidity as one of the favorable results from this operation.

The secretions held responsible for this chemical change are bile, the pancreatic juice, and the juice from the duodenal mucosa. It has long been recognized that varying amounts of the duodenal contents are normally present in the stomach following operations of this nature; thus Kausch, Carle and Fantino, Kaiser, Hartmann and Lecene, and numerous others have regularly observed bile in the gastric contents in these cases.

If it be true that the regurgitated duodenal or jejunal secretions exert a favorable physiological influence on the gastric contents it is likely that this change consists of a reduction of the total acidity.

Such a change might be due to either one of two factors, or to a combination of them. First, any one or more of the secretions normally present in the duodenum on reaching the stomach might lead to a reflex decrease in the production of gastric juice. Second, since the duodenal contents are alkaline, any one or more of these secretions might neutralize the acid chyme of the stomach.

The purpose of the investigations reported here has been to determine if possible whether bile when it is introduced into the stomach plays a significant part in reducing the acidity of the gastric contents. The importance of the pancreatic and duodenal secretions in this respect have been left for further studies.

HISTORICAL.

The effect of the bile salts on gastric secretion has been studied clinically by Glaessner. After feeding sodium choleate to patients with hyperchlorhydria he noted a definite decrease in the acidity of the stomach contents.

Palfrey reports favorable results from feeding salol-coated pills of ox bile to patients with hyperacidity. He attributes this improvement to a neutralization by bile of the acid stomach contents when they reach the duodenum. That both human and bovine bile stimulate the secretion of bile has been shown by Pfaff and Balch.

Boldyreff showed experimentally that regurgitations of pancreatic juice and bile occur regularly after the ingestion of much fat, and in the presence of a large amount of free acid—hydrochloric and fatty—in the stomach, and suggests that the backward flow of alkaline duodenal contents may represent an attempt on the part of the body to neutralize the unusual acidity of the stomach.

Opposed to the findings of Glaessner are the results from certain experiments performed by Lönnqvist. He fed dogs prepared with Pawlow pouches with bile obtained from other animals having biliary fistulæ and found that it stimulated the secretion of gastric juice to the same degree as water; that is, the acidity and the digestive power of the juice collected after the ingestion of bile corresponded closely to the acidity and the digestive power of that obtained after the injection of water. The contents of the main stomach, however, subsequent to the ingestion of bile showed almost no digestive power, due to the precipitation of the pepsin by the bile.

Wiedemann anastomosed the gall bladder to the stomach in a dog and studied the gastric contents by means of a gastric fistula. While single examinations occasionally showed a slight decrease in the total acidity, others revealed an occasional increase. He concludes that the acidity of the gastric contents, at least when milk and eggs are fed, is not appreciably affected by diverting the bile into the stomach.

The effect of bile on the gastric mucosa has been studied by a number of workers. Rywosch concluded from feeding bile to chickens and rabbits that the introduction of large amounts might be very injurious to the stomach, whereas small amounts probably produce no ill effects. In the higher animals there is considerable evidence to show that bile exerts no injurious influence on the normal mucosa. Oddi, Dastre, and Smith diverted the bile from the duodenum into the stomach in dogs and noted no pathological changes in the gastric wall. Oddi also fed large amounts of ox bile to dogs for many days with similar results. In their cholecystgastrostomy experiments Dastre and Wiedemann analyzed the gastric contents and found digestion unaffected by the bile. Smith emphasizes the importance of mucus as a means of protecting the stomach against the action of chemical agents. While the introduction of weak hydrochloric acid and bile separately never led to lesions of the mucosa, combinations of the two, when injected into the stomach during the course of digestion (mucus being probably less abundant at this time), usually resulted in some necrosis of the epithelium.

Cannac, Masse, Mocquot, and others have reported an absence of ill effects following the anastomosis of the gall bladder to the stomach in dogs. Clinical experience has afforded similar results in man. Kehr, Jaboulay, Eichmeyer, Jacobson, and numerous other surgeons have found this operation in many instances to be preferable to cholecystenterostomy.

Methods Employed.

While Chigin has shown that the course of secretion in the smaller Pawlow stomach exactly corresponds to that in the larger, it was thought that by following the gastric secretion in both stomachs before and after the administration of bile each set of experiments would serve as a check on the other. Accordingly the work was divided into two parts. In one series of animals pure gastric juice was obtained from Pawlow pouches. So far as can be ascertained this method has not been used for investigating the gastric acidity in the presence of a cholecystgastrostomy. In the other series the gastric contents were obtained by injecting apomorphin hypodermically, as suggested by Hamburger and Friedman in their work on pyloric stenosis. Analyses were made throughout the various stages of digestion by repeating the test meal on different days, the stomach being emptied each day at a different interval after the ingestion of the food. An interval of one or more free days was always allowed between such analyses.

Great care was taken in forming the miniature Pawlow stomachs to prepare with a broad base the flap of the stomach wall used for

the pouch so that the pedicle of muscularis and peritoneum might carry an ample nerve supply to the pouch. It was found that the erosion of the abdominal wall about the pouch opening could be greatly minimized by leaving a full cuff of mucosa projecting above the surface of the abdomen. From a series of more than a dozen dogs prepared in this manner three were found to be satisfactory in every way for the investigation.

Bile was supplied by ligating and dividing the common duct and anastomosing the gall bladder with the stomach. The normal color of the stools, the absence of jaundice, and the patency of the anastomosis at autopsy proved that all of the bile had entered the alimentary tract via the stomach.

The operations were conducted under full ether anesthesia. In the animals reported here the wounds all healed by primary intention.

The analyses were made with dimethylamidoazobenzol and phenolphthalein. In the tables the results are expressed in terms of cc. of N/10 sodium hydroxide—acidity per cent. Ten minutes after the ingestion of the test meal the dog was placed in its canvas hammock and a collection of gastric juice was started. Analyses were carried out at fifteen minute intervals.

It is hardly necessary to add that an ample period of time was allowed after each of the operations in order that the animals might completely recover before the secretory activity of the stomachs was investigated.

The Effect of Cholecystgastrostomy on Gastric Acidity.

The Quantity and the Acidity of the Gastric Juice.—Table I is representative of the results obtained from following the gastric secretion by means of Pawlow pouches. Numerous examinations were made both before (controls) and after the diversion of the bile stream into the stomach. While slight variations from the controls were noted at times after the cholecystgastrostomies the minor degrees of hypoacidity were fully counterbalanced by slight degrees of hyperacidity. As a whole this method yielded very constant results.

The figures in Experiments IV and XIII are from the protocol of Dog II. Those in Experiment XIII were obtained four weeks sub-

sequent to the ligation and division of the common bile duct and the anastomosis of the gall bladder to the stomach. On comparing the two tables it is evident that there was very little difference in the amounts of gastric juice secreted from the small stomach before and after the operation. During the last hour and a half of the collections the total acidity was somewhat lower in Experiment XIII. This feature, however, was not constant throughout the experiments, but was found to vary with the period of convalescence. Where the curve of secretion was investigated soon after the cholecystgastrostomy (three to four weeks), figures were obtained such as are recorded

TABLE I.

Dog II.

Experiment IV.*					Experiment XIII.*				
Before cholecystgastrostomy.					After cholecystgastrostomy.				
Time.	Juice.	Free acidity.	Combined acidity.	Total acidity.	Time.	Juice.	Free acidity.	Combined acidity.	Total acidity.
	cc.					cc.			
7.45	5.5	110	10	120	7.15	7.0	105	10	115
8.00	10.0	140	5	145	7.30	9.0	135	5	140
8.15	6.0	145	5	150	7.45	10.5	150	5	155
8.30	6.0	140	5	145	8.00	6.5	145	5	150
8.45	5.0	135	10	145	8.15	4.7	140	5	145
9.00	3.2	130	10	140	8.30	3.3	125	10	135
9.15	4.0	130	10	140	8.45	3.1	130	5	135
9.30	4.0	125	10	135	9.00	3.0	120	10	130
9.45	3.0	110	10	120	9.15	3.0	115	10	125
10.00	2.6	125	5	130	9.30	2.8	115	10	125
10.15	3.0	120	10	130	9.45	2.5	120	10	130
10.30	3.1	120	5	125	10.00	2.6	115	5	120
10.45	2.7	120	10	130	10.15	2.5	105	10	115
11.00–11.15	3.4	115	10	125	10.30	2.2	100	10	110
					10.45	2.0	95	10	105
11.30–11.45	2.7	115	5	120	11.00–11.15	3.1	95	5	100
12.00–12.15	1.0	110	5	115	11.30–11.45	2.0	90	10	100

* Test meal: chopped meat 150 gm. and water 140 cc.

here. Where analyses were made at later periods, on the other hand, the acidity values rose to a higher level. Two and one-half months subsequent to the operation no appreciable difference was noted in the figures obtained during the last hour and a half of the collections when these were compared with those secured previous to the gall bladder anastomosis. At this later date, nevertheless, during the first three hours of digestion a very slight rise in the total acidity was frequently found.

These results indicate then that bile when present in the stomach in considerable amounts during digestion exerts no appreciable influence on the secretion of gastric juice, and has no significance in decreasing the acidity of the gastric contents, at least with a diet of meat and water. While it is problematical just what quantity of bile reaches the stomach during this phase of digestion in such animals, it appears likely that the acid chyme entering the duodenum serves to activate the prosecretin as usual, and in this way stimulates a more or less normal secretion (Howell).

Foster and Lambert have called attention to the slight individual variations noted among dogs fed on the same kind of food, both in the amounts of secretion and in the percentage content of acid. They conclude that the only evidence of limitation of function that we can gain must depend, to be of value, upon an average computed from the data for a number of animals. In the experiments reported here this feature has no essential bearing on the results since comparisons of the data from one animal were not made with those from another. In each dog an average of the findings obtained during a number of tests before cholecystgastrostomy was contrasted with that obtained from a similar series of experiments conducted subsequent to the operation. As a matter of fact the data from the three animals showed very slight differences.

Each of the three dogs reacted well to the cholecystgastrostomy. There was no appreciable vomiting at any time and in each instance on the day following the operation the animal drank its allowance of broth. During the course of the experiments the stools remained colored and of normal consistency. The general nutrition of the animals continued good throughout the following months. In one instance the observations extended over six months.

The Acidity of the Gastric Contents.—The figures in Table II are from the protocol of Dog VI. They illustrate the average results obtained from following the acidity of the gastric contents before and after the diversion of the bile stream into the stomach. Here again subsequent to the cholecystgastrostomy variations from the controls were noted, both decreases and increases in the total acidity. These counterbalanced one another well, however, showing no appreciable average deviation from the normal. As a whole, the fluctuations in acidity were somewhat greater with this method.

TABLE II.

Dog VI.

Experiment II.*				Experiment V.*			
Before cholecystgastrostomy.				After cholecystgastrostomy.			
Hours after feeding.	Free acidity.	Combined acidity.	Total acidity.	Hours after feeding.	Free acidity.	Combined acidity.	Total acidity.
1	0	56	56	1	0	65	65
2	0	49	49	2	8	62	70
3	18	50	68	3	21	68	89
$4\frac{1}{4}$	25	46	71	$4\frac{1}{4}$	25	40	65
5	21	69	90	5	16	87	103
6	32	42	74	6	31	41	72
7	20	38	58	7	17	50	67
$8\frac{5}{12}$	15	30	45	$8\frac{5}{12}$	9	29	38

* Test meal: chopped meat 100 gm. and water 100 cc.

Experiment II presents the data secured about three months subsequent to the anastomosis of the gall bladder with the stomach. At this postoperative period the analyses showed a slightly increased acidity of the gastric contents as compared with the data obtained previous to the cholecystgastrostomy. The same inclination toward a hypersecretion was noted in the other two animals at corresponding periods. The results were a little different, however, when the analyses were made at shorter intervals after the operations. At the end of four or five weeks the acid values averaged slightly lower than those secured with the stomach in its normal state. The acidity of the gastric contents then increased somewhat as the weeks passed.

In no dog of the series was any marked or permanent decrease in the acidity found.

As a rule the test meals secured after the cholecystgastrostomies closely resembled macroscopically those obtained previous to the operations, except for a slight yellowish tint of the fluid portions. Occasionally a trace of free bile was evident.

Two of the dogs showed some temporary effects from the anastomoses. There was urgent emesis during the first twenty-four hours, and an occasional emptying of the stomach in the course of the next few days. With this was coupled a striking anorexia. As a result the nutrition of both animals was decidedly affected. During the second week, however, as the appetite improved each dog put on weight and became more active. The third dog manifested no ill effects of any kind. Throughout the course of the experiments the stools remained colored and of normal consistency. Three or four weeks after the operations the dogs were all well nourished and showed no disturbances of their gastro-intestinal tracts.

Postmortem examinations in both series of animals showed an obliterated common bile duct with a patent communication between the gall bladder and the stomach in each instance. The gastric mucosa showed no pathological changes.

SUMMARY AND CONCLUSIONS.

It has been shown that small amounts of the digestive secretions normally present in the duodenum regurgitate into the stomach following operative anastomosis between the stomach and the upper intestinal tract. As the duodenal contents are alkaline in reaction this reflux is thought by many to result in a decrease in the acidity of the gastric contents.

The purpose of the investigations reported here has been to determine the effect of bile on the acid chyme of the stomach. By means of a cholecystgastrostomy with ligation and division of the common bile duct in six dogs the bile was diverted from the duodenum into the stomach. The secretion of gastric juice and the acidity of the gastric contents throughout the period of digestion were followed before and after the anastomoses. For this purpose three of the dogs were

provided with Pawlow stomach pouches. In the three remaining animals the test meals were recovered by using apomorphin injections.

The results from the two series of experiments agree. They indicate, at least with a diet of meat and water, that bile when it is present in the stomach throughout the course of digestion has no appreciable effect on the acidity of the gastric contents.

While the anastomosis of the gall bladder to the stomach occasioned some temporary anorexia and vomiting in two of the dogs, the four remaining animals bore the operation well. Three or four weeks subsequent to the operation the digestion and the nutrition appeared normal in each instance.

Postmortem examinations revealed no pathological changes in the gastric mucosa.

BIBLIOGRAPHY.

Boldyreff, W., Der Übertritt des natürlichen Gemisches aus Pankreassaft, Darmsaft und Galle in dem Magen, *Arch. f. d. ges. Physiol.*, 1908, cxxi, 13.

Cannac, L. B., De la cholécystogastrostomie, Thèse de Bordeaux, 1897.

Carle, A., and Fantino, G., Beitrag zur Pathologie und Therapie des Magens, *Arch. f. klin. Chir.*, 1898, lvi, 1.

Collinson, R., Some Present-Day Problems in the Surgery of Gastric and Duodenal Ulcers, *Jour. Am. Med. Assn.*, 1914, lxiii, 1184.

Dastre, A., Recherches sur la bile, *Arch. de physiol. norm. et path.*, 1890, series 5, ii, 315.

Eichmeyer, W., Beiträge zur Chirurgie des Choledochus und Hepaticus einschl. der Anastomosen zwischen Gallensystem und Intestinis, *Arch. f. klin. Chir.*, 1910, xciii, 857.

Finney, J. M. T., and Friedenwald, J., Thirteen Years' Experience with Pyloroplasty, *Surg., Gynec. and Obst.*, 1914, xviii, 273.

Foster, N. B., and Lambert, A. V. S., Some Factors in the Physiology and Pathology of Gastric Secretion, *Jour. Exper. Med.*, 1908, x, 820.

Glaessner, K., Eine neue Therapie der Hyperazidität des Magens, insbesondere bei ulzerösen Prozessen, *Wien. klin. Wchnschr.*, 1913, xxvi, 1557.

Hamburger, W. W., and Friedman, J. C., Contributions to the Experimental Pathology of the Stomach. I. Experimental Pyloric Stenosis, *Arch. Int. Med.*, 1914, xiv, 722.

Hartmann, H., and Lecene, P., Ulcer of the Stomach and of the Duodenum, *Ann. Surg.*, 1914, lx, 227.

Howell, W. H., A Text-Book of Physiology, 5th edition, Philadelphia and London, 1913, 803.

Jaboulay, La cholécystogastrostomie pour les tumeurs de la téte du pancréas, *Lyon méd.*, 1898, lxxxix, 365.

Jacobson, J. H., Anastomosis of the Gall-Bladder to the Stomach: "Cholecystogastrostomy," *Tr. Am. Assn. Obst. and Gynec.*, 1914, xxvii, 172.

Kaiser, O., Über die Erfolge der Gastroenterostomosis, *Deutsch. Ztschr. f. Chir.*, 1901, lxi, 294.

Kausch, W., Ueber funktionelle Ergebnisse nach Operationen am Magen bei gutartigen Erkrankungen, *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1899, iv, 347.

Kehr, H., Die Praxis der Gallenwege-Chirurgie in Wort und Bild, Munich, 1913, ii, 67.

Lönnqvist, B., Beiträge zur Kenntniss der Magensaftabsonderung, *Skand. Arch. f. Physiol.*, 1906, xviii, 194.

Masse, M., Cholecystogastrostomie, *Rev. de chir.*, 1898, xviii, 1122.

Mayo, W. J., Chronic Ulcers of the Stomach and Duodenum, *Ann. Surg.*, 1914, lx, 220.

Mocquot, P., Anastomoses de la vésicule biliaire avec l'estomac et avec le duodenum, *Compt. rend. Soc. de biol.*, 1911, lxxi, 118.

Noetzel, A., Communication to Eliot, *Ann. Surg.*, 1912, lv, 709.

Oddi, R., Physiology of the Digestive Glands, *Centralbl. f. Physiol.*, 1887–88, i, 312.

Palfrey, F. W., The Administration of Ox Bile in the Treatment of Hyperacidity and of Gastric and Duodenal Ulcer, *Am. Jour. Med. Sc.*, 1913, cxlv, 796.

Pawlow, I. P., The Work of the Digestive Glands, London, 1910, 108.

Pawlow, J. P., Die physiologische Chirurgie des Verdauungskanals, *Ergebn. d. Physiol.*, 1902, i, pt. 1, 246.

Pfaff, F., and Balch, A. W., An Experimental Investigation of Some of the Conditions Influencing the Secretion and Composition of Human Bile, *Jour. Exper. Med.*, 1897, ii, 49.

Rywosch, D., cited by Smith, G. M., An Experimental Study of the Relation of Bile to Ulceration of the Mucous Membrane of the Stomach, *Jour. Med. Research*, 1914, N. S., xxv, 147.

Smith, G. M., An Experimental Study of the Relation of Bile to Ulceration of the Mucous Membrane of the Stomach, *Jour. Med. Research*, 1914, N. S., xxv, 147.

Wiedemann, H., Experimentelle Untersuchungen zur Lehre der Verdauung und Resorption verschiedener Nahrungsprodukte bei anormalem Gallenzufluss in den Verdauungsapparat, *Beitr. z. klin. Chir.*, 1914, lxxxix, 594.

A PATHOLOGICAL STUDY OF SYPHILITIC AORTITIS AND ITS SEROLOGY.

By JOHN H. LARKIN, M.D., and I. J. LEVY, M.D.

(*From the Strecker Memorial Laboratory of the City Hospital, New York.*)

Plates 1 to 7.

(Received for publication, June 26, 1915.)

The Wassermann reaction as a diagnostic factor in syphilis holds a unique position in clinical medicine. There are those who believe it an infallible method for proving the presence of syphilis, but there are as many who are skeptical about accepting a serological diagnosis. Neither the serologist nor the clinician can hope definitely to ascertain the value of the complement fixation test in lues. As in all other problems in medicine, it is the work of the pathologist to demonstrate the probable focus of infection in an individual with a positive Wassermann reaction, in whom there is no clinical evidence of syphilis. With this in view it occurred to us to check the serological diagnosis on the autopsy table; that is, to find some definite pathological explanation for this type of reaction in so called latent syphilis. Since syphilis primarily attacks the blood vessels—in fact, it is regarded as a disease the manifold pathological changes of which have their origin in diseased blood vessels—we turned our attention first to the aorta.

Though the pathology of specific aortitis is fairly well understood and its various phases have been accurately described, and though there is a classical method of performing the Wassermann reaction, the problem presents many difficulties. Luetic aortitis, grossly and microscopically, presents many characteristic features, so our task is chiefly to point out what are the primary and early changes of a syphilitic process in the aorta. Since numerous modifications of the original Wassermann method are employed in doing the reaction, it is essential to study and to determine a certain routine serological

procedure, which, with the greatest degree of accuracy, will diagnose the presence or absence of specific changes in the aorta.

We obtained aortas from every case autopsied in the past four years in the Strecker Memorial Laboratory on which a serological examination of the blood had been made before death. Unfortunately, this includes only a small number of cases sectioned. With the aid of serology we have been able to point out definitely that the diagnostic lesion of specific aortitis is a histological one, and in the routine examination of a series of aortas a surprising number reveal this lesion in varying degree, without giving any gross evidence of syphilitic infection.

Gross Appearance.

In most instances in those who have died of aortitis the gross appearance of the aorta presents a typical picture. The lesion is confined to a definite portion of the aorta, usually the ascending and transverse parts, with a sharp line of demarcation between the lesion and the remainder of the aorta, which appears fairly normal (Fig. 3). The involved portion presents either a diffuse dilation or a saccular aneurysmal pouch, and the normal glistening appearance of the intima is lost, being replaced by confluent, pearly white, elevated scar areas, scattered through which are often seen yellowish patches of fatty degeneration (Figs. 1 and 2). To the touch it is hard, thickened, and fibrous. Though this gross appearance is characteristic, it is by no means diagnostic. In our series of seventeen cases eleven died of aortitis, and a little over one-half of these presented this typical picture; the others showed either a diffuse process, the dominating feature of which was an athero-degenerating sclerosis, superimposed upon a syphilitic process, or only slight changes in the intima were appreciable.

Microscopical Changes.

Chiari, in 1903, gave an excellent description of the microscopical changes. He calls attention primarily to the striking appearance of the media, in which one notes small areas of granulation and fibrous tissue, often with central necrotic zones and newly formed vascular elements, surrounded by infiltrating round cells. He also pointed out that in the adventitia the vasa vasorum were

distinctly thickened, and that a perivascular, round cell infiltration was associated with these changes in the adventitia. He regarded the changes in the intima as secondary, and presenting nothing characteristic of a luetic process, but showing various degrees of a simple atherosclerosis. Chiari's description, however, is only typical of an advanced case of luetic aortitis. It is the histological picture seen in the aortas taken from those who have died of the disease. We feel confident, from a study of the material at hand, that an earlier process of luetic aortitis presents quite a different picture. A productive inflammation in the aorta to our mind is pathognomonic of syphilis, and histologically it can easily be differentiated from the simple degenerating process in atherosclerosis. The earlier changes we have seen in aortas from individuals dying of tabes or syphilitic meningomyelitis, and, in several instances, from a non-syphilitic disease. The adventitia, undoubtedly, is the site of predilection, and here one sees the earliest evidence of a productive inflammation. Grouped around the slightly thickened vasa vasorum there is a distinct round cell infiltration. Associated with this perivascular inflammatory reaction the adventitia appears thickened; the fibers are coarser and heavier, and the thickening is directly proportionate to the degree of infiltration. It is important to note in this early stage that the inflammatory process is usually confined to the adventitia, and that the medial coat presents no evidence of a fibrous or lymphoid infiltration. In many instances, however, with a moderate perivascular infiltration in the adventitia the media and intima may be the seat of a marked degenerative process.

From a thorough study of a number of syphilitic aortas it is evident that a perivascular round cell infiltration is a constant feature of the diseased process. The typical cells closely resemble lymphocytes, small round cells staining intensely with a nuclear stain. With these one sees other types,—endothelial and plasma cells—but these are by no means invariably present (Fig. 7). Many have found giant cells in the infiltrations in the media, and a few have called attention to their presence in the adventitia, but in our series we were unable to observe any in our microscopical preparations. Gruber seldom found them and agrees with Thorel, that one may be unable to see giant cells, even in the examination of a large number of cases.

Perivascular Cellular Infiltration as a Diagnostic Feature of Luetic Aortitis.

That this histological lesion in an aorta, namely, perivascular cellular infiltration, has syphilis as its etiological factor, and further, that it is diagnostic of luetic aortitis, we base our opinion upon the following facts.

Eighteen of the forty-two aortas examined presented this picture, and seventeen of these gave a positive Wassermann during life. In other words, 94 per cent of the aortas showing a round cell infiltration gave a positive Wassermann reaction. On the other hand, twenty-four aortas presented no evidence of a perivascular infiltration, and twenty-two of these gave negative, while two gave positive reactions; that is, 91 per cent of these aortas gave negative complement fixations. Gruber working with postmortem serum in a series of 106 cases of syphilitic aortitis obtained identical results, 94.3 per cent positive fixations, and he states that any evidence of a productive inflammation in the aorta with a positive Wassermann gives us at least 0.9 per cent assurance that this type of inflammation is a result of syphilitic infection, even though the dominating feature is one of deforming atherosclerosis.

Such lesions as ruptured aneurysm, and aortitis with pure aortic lesions, undoubtedly syphilitic in origin, histologically present this in a marked degree. In other general metabolic and infectious processes, such as arteriosclerosis, interstitial nephritis, chronic ulcerative tuberculosis, carcinoma, pneumonia, vegetative endocarditis, and pernicious anemia, the aortas showed no such histological picture.

Most authorities, however, do not agree that a perivascular, round cell infiltration in the aorta is characteristic and pathognomonic of luetic infection. Stadler maintains that moderate infiltration around the vasa vasorum may be due to alcohol and to certain infections, and Mönckeberg describes it in a case of recurrent endocarditis with rheumatic joint lesions. Gruber admits that syphilis can cause only a productive inflammation with the typical gross changes in an aorta, but that an inflammatory infiltration may occasionally, though seldom, be caused by other processes, and while histologically he is unable to determine the etiological factor, the serological reaction gives him the diagnosis. Gruber agrees with Faber, that though the dominant feature of atherosclerosis is a degenerative process with calcification, ulceration, and deformity, undoubtedly a moderate inflammatory reaction is occasionally met with in this type of sclerosis. We will admit that fibrous connective tissue and thickened vasa vasorum surrounded with fibroblastic cells are occasionally seen in atherosclerosis, and that this is a chronic productive inflammation,

but that the typical round cell perivascular infiltration, already described, is a luetic process.

That infectious diseases, such as colon bacteremias or streptococcic endocarditis, are a possible cause of lymphoid infiltration in an aorta does not coincide with our experience. In two cases of vegetative endocarditis, and in one of pyonephrosis with colon infection, the aortas showed no inflammatory changes, and the serology was negative. In another, however, with focal necroses (a streptococcic infection) the aorta showed moderate perivascular infiltration, but the serology was positive, and the infection was secondary to an ulcerating gumma of the larynx. Gruber has noted two interesting cases of inflammatory infiltrations around the vasa vasorum in the adventitia of the aorta, secondary to an inflammatory process in the para-aortic tissue. In one case tubercular lymph glands in the mediastinum had ulcerated, producing a marked inflammatory reaction. In the other, perforation of a stenosis of the esophagus through surgical interference, from which the patient died several months later from sepsis, produced a typical productive inflammation in the aorta, which could not be differentiated from a luetic process. Both these cases gave negative Wassermann reactions, and Gruber concludes that they were non-specific. That a lymphoid infiltration and productive inflammation may result as an extension process is undoubtedly true, but that does not alter our opinion that this type of reaction produced in an aorta *per se* is pathognomonic of syphilis.

Treponema pallidum in Luetic Aortitis.

Though serology gives us definite proof that syphilis produces a typical aortitis, as further evidence in support of our view, we have attempted to find *Treponema pallidum* in our preparation. Wright and Richardson, with others, claim to have demonstrated the spirochæta in the aortas of acquired syphilis, but we have been unsuccessful in doing so. We have seen many artefacts in our preparations, which, by less skeptical men, could easily be interpreted as spirochætæ. Both Gruber and Fukushi have also failed to find the spirillum in their large series of cases.

Classification of Cases.

So, basing our diagnosis upon a round cell perivascular infiltration we have in our series eighteen cases showing evidence of syphilitic aortitis. We have divided this series into three classes: first, those showing this in a marked degree; second, those cases in which the infiltration is moderately present; and third, those in which there is only slight evidence of a cellular infiltration (Figs. 4, 5, and 6). In Class 1 there are eleven cases. Over one-half of the aortas presented the gross picture of specific aortitis; the rest showed a diffuse atheromatous process, with areas of ulceration. All these cases died of aortitis; three with ruptured aneurysm, the others with symptoms of cardiac decompensation. Five of the cases had marked aortic valve involvement, with normal mitral valves, or slightly thickened mitral cusps. One case had both valves involved. There was positive serology in all cases.

In Class 2, a series of five cases, none presented the gross typical picture. Three of the aortas were markedly atheromatosed, with areas of calcification and ulceration. The two others were fairly normal, with the exception of longitudinal striations and thickened, raised, whitish areas around the intercostal orifices. Histologically, however, the perivascular infiltration was distinctive and definite. In only one case was the aortic valve diseased. None of these cases died of their aortitis. One died with symptoms of cardiac decompensation, probably from localized fibrous infiltration of the wall of the left ventricle, involving the area of the bundle of His, with aneurysmal dilatation at this point. The others died of the following conditions: syphilitic meningomyelitis, carcinoma of the right bronchus, chronic ulcerative tuberculosis, and terminal bronchopneumonia with tabes. The serology was positive in all but one case.

In the third class, consisting of only two cases, characterized by a very moderate or slight infiltration around the vasa vasorum the aortas appeared practically normal, with the exception of patches of smooth, elevated areas around the intercostal orifices. One case died of carcinoma of the bronchus, the other of an ulcerating gumma of the larynx, with focal necroses of kidney and liver. Both cases had a positive serology.

Complications.

The most common complication of syphilitic aortitis is an insufficient aortic valve. In a study of forty-two cases with various types of aortas the aortic valve in fifteen showed evidence of disease. In seven of these fifteen valvular lesions the mitral valve was normal, and the aortas of these seven cases, in which only the aortic valves were involved, gave marked evidence of luetic aortitis, and the serology was positive, with the exception of one. In this case the aortic valve was practically destroyed with a vegetative ulceration; the serum was negative and the aorta normal. Of the remaining eight cases both valves were diseased; six consisting of fibrous changes, and two having acute vegetations. In only one aorta with thickened fibrous changes in both valves was there evidence of luetic aortitis, and in this case also the serum was positive. The seven other cases showed no productive inflammation in the aorta, and the complement fixation tests were negative. From this it seems evident that pure aortic insufficiency, with the exception of infectious endocarditis, is undoubtedly of syphilitic origin. With both valves involved the probability of a syphilitic infection of the aorta is small; an atheromatous or an infectious process should suggest the probable origin.

Luetic aortic insufficiency is, in most cases, probably a late process. In our seven cases of syphilitic involvement of the aortic valve, six were a complication of those specimens which showed marked histological changes in the aorta (Class 1) and all of these died of the vascular lesion. In other words, six out of ten specimens of late aortitis, or 60 per cent of the cases of advanced aortitis, had the aortic valve involved. On the other hand, of the seven cases of early luetic changes the aortic valve was part of the process only once, giving in our whole series the complication in about 40 per cent of the cases. This practically agrees with Stadler's findings, in which he states that while only one-third of his luetic aortas were complicated with a diseased valve, two-thirds of his cases dying of aortitis had insufficient aortic valves..

Ruptured aneurysm is a serious, though not so frequent a complication. Of course it is met with only in advanced cases. In our series of eleven dying with aortitis, three succumbed to a rupture of

an aneurysm. In two the rupture was in the thoracic cavity. In one the aneurysm was abdominal and had eroded the vertebra almost to the cord.

Cerebral luetic endarteritis is a complication of syphilitic aortitis as often as is aortitis. We can only consider cerebral endarteritis a complication of the aortitis when there are symptoms of a brain lesion, and evidences of brain softening in an individual dying of aortitis. Conversely, in individuals dying of their brain lesions the endarteritis is complicated with an aortitis, when there are either clinical or pathological evidences of aortic disease present. In our eleven cases of fatal aortitis two individuals had evidences of paralysis before death, and in these cases areas of brain softening were demonstrated. In seven cases of early aortitis only one died of cerebral softening.

Tabes complicates specific aortitis less often than it is complicated by the vascular lesion. In over ten fatal cases none gave during life any clinical evidence of tabes. It is of interest to note here, however, that the histological examination of the cord in one case revealed an early sclerosis of the posterior root fibers, the posterior tract of the cord not being affected. In two fatal cases of tabes diffuse atheroma with calcification and ulceration were present in both aortas. In one, however, there was a moderate perivascular infiltration, and the serum in this case was positive; the other gave no evidence of syphilis in the aorta or in the serum. Stadler found clinically in 248 cases of aortic disease that 6.2 per cent of these had tabes, and he further states that in his autopsy findings almost all cases of tabes showed syphilitic aortitis.

Aortitis is a frequent complication of general paresis. Gruber and Straub at autopsy found that 71 and 82 per cent, respectively, of their cases of general paresis showed evidence of luetic aortitis.

Stadler found narrowing of the lumen of the mouth and sclerosis of the coronaries in one-third of his autopsies on aortitis. This is undoubtedly true, but it is difficult to differentiate the dominating factor producing this change, whether an atherosclerosis or a productive inflammation. It is questionable, as the older writers have suggested, whether the earliest changes in syphilis of the aorta are seen around the orifice of its branches, producing a narrowed lumen.

Only occasionally have we noticed in advanced aortitis anything approaching a definite stenosis of these orifices.

In over two-thirds of our cases there was no cardiac enlargement (Figs. 1 and 2). This agrees with Grau's findings, but Gruber in a majority of his specimens reports a distinct hypertrophy of the heart. Some have stated that cardiac hypertrophy is associated with the aortic insufficiency, but in some of our most marked cases of aortic disease we have found surprisingly small hearts. It seems, however, that cardiac hypertrophy is not associated with luetic aortitis or any of its complications, but is a result probably of some type of nephritis.

Serology.

To discuss technically the serology of syphilitic aortitis is not the scope of this paper, but rather to point out that in our hands, at least, a definite serological procedure is preferable to either the original method, or several popular modifications, in diagnosing syphilis of the aorta, and that a high percentage of positive reactions in individuals who give no clinical evidence of syphilis of the vascular system can be explained by luetic aortitis.

Wassermann Reaction as a Diagnostic Factor in Luetic Aortitis.

As stated, forty-two aortas have been examined and the sera of these cases tested serologically before death. Nineteen of these gave positive Wassermann tests; twenty-three were negative. Of the nineteen positive cases, seventeen aortas gave microscopical evidence of luetic aortitis; namely, 90 per cent of our positive reactions (those sera fixing complement according to the method above described) have, at least, a definite pathological explanation based on changes in the aorta which are pathognomonic of syphilis. It is interesting to note that fifteen of the nineteen cases died as a result of a syphilitic process (about 80 per cent), while eleven of the nineteen died from their luetic aortitis (about 60 per cent). The four cases dying from syphilis other than aortitis were one case of syphilitic cerebral endarteritis, one of tabes, one of meningomyelitis, and one ulcerating gumma of the larynx. Of the four cases with positive Wassermanns dying of non-syphilitic conditions but showing perivascular infiltra-

tion in the aorta, two died of carcinoma of the bronchus, one of chronic ulcerative tuberculosis, and one of interstitial nephritis.

Though the cases studied are too few to make any definite deduction, they are suggestive. It is highly probable that about 90 per cent of individuals dying with a positive Wassermann, if the infection is not of recent origin, have, at least from the histological point of view, luetic aortitis, that about 60 per cent of them die from their aortitis, giving clinical evidence of cardiac decompensation or rupture of an aneurysm, and that about 80 per cent of these individuals die of syphilis. Since only one case of perivascular cellular infiltration gave a negative Wassermann test in our series, it is fair to assume that 94 per cent of individuals suffering from luetic aortitis would give a positive reaction in their serum.

That our results correspond with the data in the literature is shown by the recent works of Stadler and Gruber. Gruber obtained identical serological reactions in his series of luetic aortitis; namely, 94.3 per cent positive Wassermanns on postmortem serum. Basing his results on the pathological findings, Stadler states that 82 per cent of 256 cases of syphilis had aortitis, and so considering a positive fixation as diagnostic of syphilis we find, as stated, that 90 per cent of syphilitics have evidence of aortitis. Our higher figure could easily be accounted for by considering (as we did) every aorta with perivascular infiltration as specific. Though only 46 per cent of Stadler's cases died of their vascular lesion, in our series we found that the aortic lesion accounted for deaths in 60 per cent.

The serology of aortic insufficiency is confusing. Serologists obtain positive reactions ranging anywhere from 50 to 80 per cent of the cases. The reason for this large discrepancy is due to the fact that the clinicians have failed to differentiate between the various types of this lesion. If one remembers that only pure aortic insufficiency, with the exception of an infectious endocarditis, is always syphilitic, and that the serology is positive in probably 100 per cent of the cases, and that the aortic lesion associated with a mitral incompetent valve is usually an atherosclerotic process, and only occasionally specific, one can readily understand the disagreement in the serological results.

Wassermann Reaction in Treated Cases.

The effect of treatment on the serology of luetic aortitis should receive passing notice. All the cases with varying amounts of treatment, including salvarsan and mercury, with the exception of four, remained positive throughout. The serology was modified in four cases, as follows: No. 292 was admitted with a positive Wassermann reaction; salvarsan was given, followed by several doses of mercury, and following this the Wassermann was negative. No. 678 was admitted with a weakly positive reaction; that is, 50 per cent hemolysis on the guinea pig antigen. Before admission the patient had received several doses of mercury. Without treatment at the end of a month the serology was strongly positive. Five salvarsan injections were given, and a month later the Wassermann test was still positive. A year later, however, just before death, the serology was reported doubtful; since, though there was complete inhibition of all antigens at the first reading, at the end of twelve hours there was complete hemolysis, even with cholesterinized antigen. No. 694 was admitted with serology noted as weakly positive, 50 per cent hemolysis on guinea pig heart antigen. Mercury had been given before admission. Eight months later (three months after the salvarsan and mercury treatment) the serum was reported positive. No. 691, which was admitted with a positive reaction after one salvarsan and several mercury injections, was reported as negative.

A careful review of these cases notes the importance in serologically diagnosing luetic aortitis of repeating the reaction on sera, especially if any form of antispecific treatment has been administered. Though in 75 per cent of the cases treatment did not alter the serological reaction in the blood, in 25 per cent it either weakened the reaction or produced a negative fixation.

Technique and Antigens.

In this series a positive reaction means complete inhibition of hemolysis when the controls have hemolyzed, and at the end of twelve hours. The antigen employed is an alcoholic extract of guinea pig heart. 0.1 cc. of complement and at least two units of amboceptor are used in the hemolytic system. Many of the sera were

done with other antigens; namely, alcoholic extract of syphilitic liver, acetone partition of calf's heart, cholesterinized alcoholic extract of human heart and guinea pig heart. But the findings with these various antigens were not as constant and as consistent as with the first named extract. We do not mean to infer that these extracts are not to be used in the Wassermann reaction, but rather that in the serological diagnosis of syphilitic aortitis, the alcoholic extract of guinea pig heart should receive first consideration.

The antigens made from syphilitic fetal livers were unreliable with us, while acetone partition antigens made according to Noguchi's method, though giving fairly good results, were influenced earlier by treatment and the completed extracts seldom were of uniform strength. These antigens have been discarded by us in reporting the routine Wassermann reaction in the laboratory. It is important to note, however, that Gruber working with fetal syphilitic liver antigens obtained results identical with ours. Our experience with the liver antigen (that it is so unreliable) is not surprising, since, though we attempted to obtain congenital luetic organs, in many instances we were not assured by spirochæta demonstration that our material was from a syphilitic infant. The highly sensitized cholesterin antigens we still employ with the assurance that complete complement fixation with this antigen probably does not always indicate the presence of syphilis. On the other hand, however, many other types of syphilis, such as tabes, give only positive reactions with this antigen.

Though 90 per cent of our positive reactions with guinea pig heart antigen showed evidence of syphilis in the aorta, only 77 per cent of the positive results with cholesterin could be explained by similar luetic changes.

Finally, we hope that these facts will be of assistance to the clinician in diagnosing syphilis of the aorta, and further, that they will emphasize the importance of a positive Wassermann reaction, and make it incumbent upon all physicians to institute treatment as a prophylactic measure in such cases.

Syphilis is an endemic infectious disease which ranks with tuberculosis and carcinoma as a menace to the community, and which, when untreated, probably kills 80 per cent of its victims and produces an aortitis in about 80 to 90 per cent, of whom 50 or 60 per cent die of the vascular lesion.

SUMMARY.

1. Syphilitic aortitis is a productive inflammatory process, the earliest and most constant feature of which is a perivascular round cell infiltration in the adventitia.

2. The typical gross picture of luetic aortitis is often obscured by a superimposed, diffuse atherosclerosis. In the early cases the aorta appears fairly normal, presenting only the characteristic histological changes.

3. A pure aortic insufficient valve, with the exception of an infectious endocarditis, is always luetic.

4. Cardiac hypertrophy is not a complication of luetic aortitis. When present it is usually associated with a nephritis.

5. The demonstration of *Spirochæta pallida*, even in advanced specimens of syphilitic aortas, is doubtful.

6. An antigen prepared from alcoholic extract of guinea pig heart with the original Wassermann technique should be preferred in diagnosing luetic aortitis.

7. Positive complement fixations in patients suffering with syphilis for a period of about fifteen years or longer suggest the probability, at least, of histological luetic changes in the aorta in 80 to 90 per cent of the cases. 60 per cent of these die from aortitis.

8. About 94 per cent of patients suffering with aortitis give positive Wassermann reactions.

BIBLIOGRAPHY.

Chiari, H., Ueber die syphilitischen Aortenerkrankungen, *Verhandl. d. deutsch. path. Gesellsch.*, 1904, vi, 137.

Faber, A., Arteriosklerose. Ihre pathologische Anatomie, ihre Pathogenese und Ätiologie, 2d edition, Jena, 1912.

Fukushi, M., Die pathologische Anatomie der syphilitischen Aortitis mit besonderer Berücksichtigung des Vorkommens von Plasmazellen, Berlin, 1914.

Grau, H., Über die luetische Aortenerkrankung, *Ztschr. f. klin. Med.*, 1911, lxxii, 292.

Gruber, G. B., Über die Doehle-Heller'sche Aortitis, Jena, 1914.

Mönckeberg, J. G., Ueber die Beziehungen zwischen Syphilis und schwieliger Aortensklerose vom pathologisch-anatomischen Standpunkt, *Med. Klin.*, 1915, i, 1027.

Stadler, E., Die Klinik der syphilitischen Aortenerkrankung, Jena, 1912.

Thorel, Ch., Pathologie der Kreislauforgane, *Ergebn. d. allg. Path. u. path. Anat.*, 1911, xiv, pt. 2, 681.

Wright, J. H., and Richardson, O., Treponemata (Spirochætæ) in Syphilitic Aortitis: Five Cases, One with Aneurism, *Boston Med. and Surg. Jour.*, 1909, clx, 539.

EXPLANATION OF PLATES.

Plate 1.

Fig. 1. Note the small heart with concentric hypertrophy. The aortic valves are moderately thickened. The walls of the aorta are markedly thickened. There is a diffuse dilatation of the ascending and transverse portions of the aorta, and a marked fibrous appearance of its inner wall.

Plate 2.

Fig. 2. The same as Fig. 1, but showing aneurysmal formation and involvement of the aortic valves. In both Fig. 1 and Fig. 2 the mitral valves were normal.

Plate 3.

Fig. 3. Portion of a syphilitic aorta showing a definite line of demarcation between involved and normal parts at the junction of the lower third.

Plate 4.

Fig. 4. Microscopical picture of a section of the aorta of the case shown in Fig. 1. Note the perivascular infiltration of the vasa vasorum, the thickened adventitia, the newly formed vascular elements in the media, with its round cell infiltration, also the thickened intima,—a secondary process.

Plate 5.

Fig. 5. The same as Fig. 4, but not so advanced.

Plate 6.

Fig. 6. An early process with a beginning atherosclerosis.

Plate 7.

Fig. 7. A high power picture showing the type of cells. Note the great number of lymphocytic cells and several larger plasma cells.

THE ETIOLOGY OF RAT-BITE FEVER.

BY FRANCIS G. BLAKE, M.D.

(*From the Medical Clinic of the Peter Bent Brigham Hospital, Boston.*)

PLATES 8 TO 14.

(Received for publication, September 23, 1915.)

Rat-bite fever, or Sodoku, has long been recognized in Japan as a definite febrile disease, following the bite of a rat.

Miyake (1) in 1899 was the first to describe the disease in detail as a definite clinical entity and reported eleven cases of his own with references to others in the Japanese literature. For many years reports of sporadic cases have appeared in America, the first being recorded as early as 1840 by Wilcox (2). In 1909 Horder (3) reported three cases from England, and since that time instances of the disease have been recorded with increasing frequency in the British literature. The incidence of rat-bite fever in continental Europe seems to have been less frequent. Cases have been recorded in France by Millot-Carpentier (4), De Micas (5), and Curtillet and Lombart (6); in Spain by Peña y Maya (7); in Italy by Frugoni (8); and in Germany by Schottmüller (9).

The clinical characteristics of rat-bite fever have been well presented in papers by Miyake (1), Horder (3), Ogata (10), Atkinson (11), Gouget (12), Proescher (13), and Frugoni (8). A comprehensive description of the disease with report of a new case and summary of 53 cases collected from the literature has recently been published by Crohn (14). To the cases collected by him may be added the following, making a total of 81 cases: in America, Farquhar (15)[1] 6; in the British Isles, Atkinson (11) 6; Nixon (16) 1; Dick and Rutherford (17) 1; in France, Curtillet and Lombart (6) 1; in Spain, Peña y Maya (7) 1; in India, Dalal (18) 1; in Japan, Ogata (19, 20) 9; and in Germany, Schottmüller (9) 1.

None of these cases with the exception of that reported by Schottmüller (9), which will be discussed below, has added anything new to our knowledge of rat-bite fever, so that it does not seem of value to review them in detail.

In view of the recent publication by Crohn (14) the clinical features of rat-bite fever will be given only in brief and the more detailed discussion will be devoted to the etiology and pathology of the disease.

Rat-bite fever is a paroxysmal febrile disease of the relapsing type following the bite of a rat. The wound heals readily, but after a variable incubation period of a few days to a month the wound becomes inflamed and painful. Lymphangitis and adenitis set in and are quickly followed by symptoms of systemic infection

[1] Farquhar's report is too brief to make the diagnosis certain in his cases.

ushered in by a chill and rapid rise in temperature. There is extreme prostration, severe generalized muscular pain, headache, weakness, and loss of appetite. Stupor, delirium, and even coma may supervene. There is muscular tenderness and rigidity and the tendon reflexes are frequently exaggerated. A characteristic exanthem of bluish red, erythematous, sharply marginated macules appears, varying in size from 1 to 10 cm. in diameter and of general distribution. After 5 to 9 days the temperature falls by crisis accompanied by a drenching sweat and all symptoms subside. The disease then assumes the relapsing type with paroxysms occurring at fairly regular intervals, usually about once a week. The course may vary from one to three months or even longer. Gradually the relapses become less frequent and less severe and the disease often terminates with an abortive paroxysm. The more important complications are nephritis, severe anemia, and emaciation. About 10 per cent of the cases terminate fatally, usually during the first febrile period, occasionally later from nephritis or exhaustion.

That rat-bite fever is an infectious disease dependent upon the introduction of a specific organism or virus at the site of the bite seems highly probable. There has been no definite evidence presented to show that the rat inflicting the bite was in any way diseased or other than a normal rat. In view of this negative evidence it does not seem improbable that the etiological agent is simply a saprophyte in the mouth of the rat. Individual susceptibility seems to play a definite part in the contraction of the disease, as instances are recorded in which the second of two people bitten by the same rat has been the only one to develop rat-bite fever (Miyake (1)). Nixon (16) has reported a case similar to rat-bite fever following the bite of a ferret, and Schottmüller (9) a case incident on the bite of a South African squirrel. That the etiological factor in such cases is closely related to that occurring in rat-bite fever is possible.

The relapsing type of the fever has suggested that the disease may be of protozoan or spirochetal origin, but there are no facts to substantiate this supposition.

Most of the cases reported during the last decade have been carefully studied bacteriologically without yielding definite results until the recent case of Schottmüller (9) reported in 1914. Ogata (10, 19) in a series of eight cases has described parasitic organisms in the blood, excised lymph nodes, and smears from the local lesions. He considered the organism to be a sporozoon and has observed various stages in its development,—sporozoites, merozoites, etc. Guinea pigs and rabbits inoculated with material from his cases died in 18 to 21 days. The same organism was observed in the blood and smears from lymph nodes, liver, and spleen of the experimental animals. In a subsequent communication Ogata (20) has reported on his findings in three additional cases. The same organisms were described in the blood and lymph nodes, and in addition mycelial threads were observed. The organism was cultivated at room temperature on gelatin and agar slants from blood obtained from the finger tips of his patients. Animal experimentations yielded the same results as in the former cases. It is to be noted, however, that he has changed the classification of his organism and con-

siders it to be an aspergillus, basing his conclusions on the cultural characteristics and appearances in smears prepared from experimental animals. Ogata's work has not been confirmed by other observers.

Middleton (21) in his case noted rod-like organisms in fresh blood smears, and one colony of a non-capsulated diplococcus appeared in an anaerobic gelatin blood culture. The organism was considered a contaminant and was not studied further.

Proescher (13) in 1911 reported finding numerous bacilli in sections from the excised wound in his case. The organisms stained bluish with Giemsa solution in tissue fixed in bichloride and alcohol, but could not be found in sections hardened in formalin. They were straight or slightly curved, from 0.5 to 1.5 microns long, and showed irregular or bipolar staining. A guinea pig inoculated subcutaneously with a portion of the excised wound after a three weeks' incubation period developed enlarged lymph nodes followed by inflammatory skin lesions. Sections of the enlarged lymph nodes showed a great number of the same bacilli that were found at the site of the bite of the patient. The organism could not be cultivated.

Schottmüller (9) has recently reported a case of rat-bite fever from which he obtained an organism in pure growth in eight consecutive blood cultures taken during a period of two months. Colonies appeared well developed in agar plates on the second or third day. The organism was cultivated on blood agar, Loeffler's blood serum, milk agar, and in human blood serum, but failed to grow on other media. The colonies on solid media were discrete, colorless, and pin-head size. In human blood serum growth appeared as a whitish floccular sediment. Microscopical examination showed long, fine, twisted threads, some homogeneous, some fragmented, some branching. They often showed spindle- or ball-shaped swellings, and coccus-like forms developed. The organism was non-motile, stained with the ordinary stains, and was tinged bluish with Gram's method. Growth occurred only at incubator temperature. The organism was not pathogenic for rabbits or guinea pigs. Schottmüller considered the organism to be a streptothrix and has called it *Streptothrix muris ratti.*

From a case in its clinical manifestations closely resembling rat-bite fever following the bite of a South African squirrel he isolated a similar organism which he has designated *Streptothrix taraxeri cepapi.* Inoculation of a monkey proved the organism to be pathogenic for that animal.

Of the pathological anatomy of rat-bite fever but little is known. Only one autopsy by Miura (22) is published. Macroscopically there was increase of the cerebrospinal fluid and injection of the pial vessels of the brain and cord. No further pathological changes were recorded and no mention was made of microscopic examination. Histological study of the excised wound in Proescher's (13) case showed a mixed inflammatory and proliferative lesion. The corium was infiltrated with numerous endothelial cells, lymphocytes, and polynuclear leukocytes. Young connective tissue elements were noted, and the whole lesion was surrounded by a wall of dense, hyaline connective tissue. Examination of excised lymph nodes recorded in a few instances has shown only hyperplasia.

Further pathological changes can only be surmised from knowledge of the clinical features of the disease. The inflammatory process at the site of the wound, the lymphangitis, and adenitis are rarely if ever suppurative in character in the absence of secondary infection. Superficial or deep ulceration, vesicle formation, and gangrene may occur at the site of the bite. The extensive and often severe systemic symptoms presumably may be the result of a profound toxemia from the elaboration of a toxin at the site of the wound, or may be due to a general invasion of the body by the etiological agent. Whichever occurs, the effects are especially shown in the severe muscular pain, wide-spread exanthem, and profound disturbance of the nervous system.

There is meager evidence in the literature pointing to cardiac complications. In Millot-Carpentier's (4) case a systolic murmur was discovered at the base of the heart and over the carotid arteries late in the course of the disease, but there is no definite statement as to the patient's previous cardiac condition. Reece's (23) patient, who had previously been well, six months after recovery was unable to walk rapidly without becoming breathless, and causing violent palpitation of the heart. There is, however, no mention of a physical examination.

The respiratory system except for an occasional mild bronchitis seems to be equally exempt. Gastro-intestinal symptoms are rarely of importance. The liver and spleen are almost never enlarged. The rather frequent occurrence of nephritis as a severe and often fatal complication indicates that the kidneys may be severely damaged.

The following case of rat-bite fever, which terminated fatally and came to autopsy, has proved of considerable interest from the standpoint of etiology and pathology.

The patient entered the Peter Bent Brigham Hospital, May 15, 1915, in the service of Dr. Henry A. Christian, to whom I am indebted for the privilege of reporting the case. I wish here also to express my thanks for many courtesies from the pathological department of the hospital.

E. S. A. A white woman, age 67 years, born in New York, dry goods merchant. The patient was referred to the medical service of the Peter Bent Brigham Hospital on May 15, 1915, by Dr. William P. Bolles, of Roxbury, Mass., with the following history.

Complaint.—Rat-bite.

Family History.—Unimportant. Habits good.

Past History.—Measles in childhood. Malaria 20 years ago. She has had fibroids of the uterus for many years. Otherwise she has been very well. She has had no symptoms of respiratory, cardiac, gastro-intestinal, hepatic, or renal disease.

Present Illness.—Two weeks ago, while taking a rat out of a trap, the patient

was bitten on the tip of the right index finger. The wound was not severe and she paid no further attention to it than to wash it in hydrogen peroxide. Two days later the finger became reddened and red streaks appeared running up the forearm. The arm and right axilla became painful. The patient consulted a physician who opened and dressed the wound and prescribed phenacetine. After four days the inflammation and pain had entirely subsided and she resumed her ordinary course of life, feeling perfectly well until 10 hours before admission to the hospital. At that time she had a severe chill which lasted about thirty minutes, suffered severe pain in the legs, arms, and sides, felt very feverish, and was completely prostrated. She was nauseated, but did not vomit. No other symptoms were noted and her condition was unchanged at the time of admission.

Physical Examination.—The patient is a well developed and well-nourished woman, very drowsy, dull, and apathetic, evidently suffering from severe pain throughout the body, especially in the legs. There is no cough or dyspnea. Temperature 101.4° F.; pulse 108.

Skin is pale and dry; no exanthem. Eyes, ears, nose, mouth, and throat are negative. Neck is readily flexed. Thyroid not felt.

The right axillary lymph nodes are enlarged, not tender, non-fluctuant. No other nodes are palpable. Lungs are negative.

The heart's action is regular. Apex impulse is not felt. There are no thrills. Left border of dulness is 11 cm. to the left of the midsternal line in the 5th intercostal space; right border is 3 cm. to the right of the midsternal line in the 4th interspace; upper border is at the 3d rib. Heart sounds are muffled. At the apex a soft systolic murmur is heard, not widely transmitted. There is no diastolic murmur. A2 is slightly accentuated, P2 normal.

Pulses are equal, regular, synchronous, of small volume and moderated tension.

Blood pressure: systolic 140; diastolic 86.

In the right lower quadrant of the abdomen there is a large rounded firm tumor (fibroid). Otherwise abdominal examination is negative.

Liver and spleen are not palpable.

On the tip of the right index finger is a small linear scar. There is no inflammation about the scar and no evidence of lymphangitis. There is muscular tenderness in both arms and legs.

Reflexes are normal.

June 15. Blood: hemoglobin 92 per cent; red blood corpuscles 4,400,000; white blood corpuscles 22,100. Smear: neutrophils 74 per cent; small mononuclears 17 per cent; large mononuclears 8 per cent; eosinophils 1 per cent; basophils 0. The red blood corpuscles appear normal. No parasites.

Urine is amber, clear, acid; specific gravity 1.024; trace of albumin; sugar 0; acetone 0; diacetic acid 0; bile 0; sediment, many white blood corpuscles and finely granular casts. No blood.

Blood culture, see below.

Phenolsulphonephthalein test: 48 per cent excreted in 2 hours.

May 16. Pulse and temperature, see Text-fig. 1. Patient is apathetic and complains of severe muscular pains; has no appetite. A blotchy, bluish red, macular, and maculopapular exanthem has appeared on the arms, thorax, thighs, and knees. The macules vary from 2 to 6 mm. in diameter, are sharply marginated, slightly raised, fade on pressure, and are neither painful nor itchy.

May 17. Condition unchanged. Tendon reflexes of arms are exaggerated. Stained blood smears and fresh smears examined with dark-field illumination show no parasites or bacteria.

May 18. Exanthem has faded somewhat. General condition is unchanged. Stool is negative. Wassermann reaction is negative.

May 19. Blood culture, see below. Blood serum does not agglutinate *B. typhosus*. Urine contains a trace of albumin; sediment, few finely granular casts.

May 20. Patient appears very sick, is very apathetic. Exanthem has nearly disappeared. White blood corpuscles 19,300.

May 24. General condition is improved. White blood corpuscles 14,700.

May 25. Patient's temperature has fluctuated considerably during the past few days. She had a severe chill followed by a rise in temperature this evening.

May 27. Condition is steadily improving. Muscular pain and tenderness are slight. No exanthem is present.

May 28. Temperature normal. White blood corpuscles 17,400.

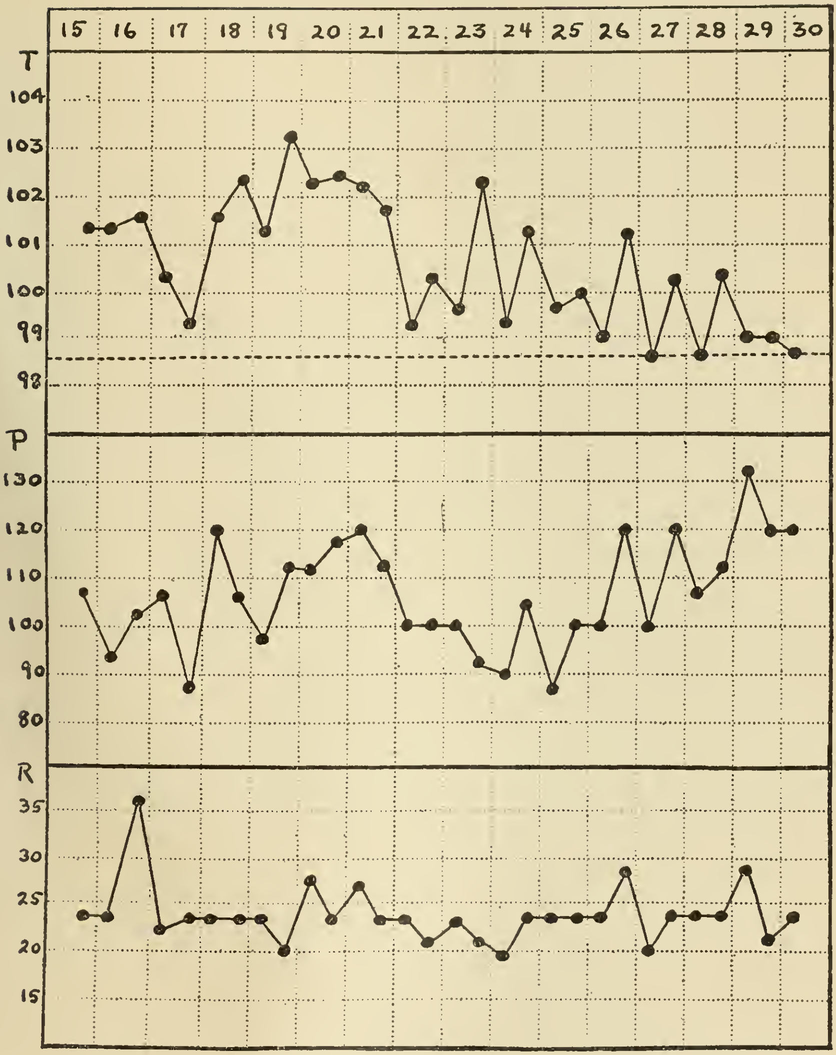
May 29. Patient was restless during the night. Pulse became rapid and weak. Patient appears very sick. Lungs are negative. The systolic murmur at the apex of the heart has become louder and harsher. A finely mottled, reddish, slightly indurated skin eruption 5 by 10 cm. has appeared on the outer aspect of the left knee.

May 30. Patient became gradually weaker and died at 7 a.m.

Autopsy Protocol.

The autopsy was done by Dr. James L. Stoddard 7 hours post mortem.

Fairly well nourished adult showing no pigmentation and no subcutaneous edema. Peritoneal cavity is free from fluid and signs of inflammation. There are several large uterine myomata. Pleural cavities contain a small amount of bloody fluid; no adhesions. Pericardial cavity contains about 5 cc. of clear yellow fluid; no adhesions. Heart (360 gm.) has a normal appearing musculature. Tricuspid, pulmonic, and aortic valves appear normal. On the posterior cusp of the mitral valve there is a large raised area made up of soft tissue, which was formed apparently under the endocardium. The area is 15 mm. in diameter and 7 mm. in height. In its center is a perfora-



TEXT-FIG. 1. Temperature chart of case of rat-bite fever (E. S. A.)

tion 4 mm. in diameter passing through the valve leaflet. The other cusps of the valve appear normal. Lungs show moderate congestion. Liver shows marked passive congestion. Spleen (200 gm.) shows a brownish red and pale mottling. On section it appears soft and edematous, the pulp scraping off easily. Two firm, pale, wedge-shaped areas are seen, with their bases at the surface of the spleen. Pancreas, gastro-intestinal tract, adrenals, and bladder appear normal. Kidneys are small (95 gm. each); surface is slightly granular; cortex 4 to 5 mm., pale gray, without distinct markings. Aorta shows marked sclerosis in the abdominal portion. Brain appears normal.

Microscopic Examination.—The myocardium shows areas of subacute myocarditis with complete disappearance of the muscle fibers, their place being taken by loose tissue filled with endothelial cells and leukocytes (Fig. 8). In the fibers elsewhere there is a high degree of fat vacuolation. Sections of the mitral valve through the vegetation (Fig. 11) show a central perforation surrounded by granulation tissue infiltrated with leukocytes. At the periphery there are masses of connective tissue, parts of which are hyalinized, parts thickly infiltrated with leukocytes, and parts necrotic. In the necrotic areas are masses of organisms appearing as long slender bacilli (Fig. 12). The muscle fibers adjacent to the lesion show all stages of necrosis.

The lung shows hemorrhage, edema, and passive congestion.

Sections of the liver show increased interlobular connective tissue, in many places thickly infiltrated with lymphocytes and polynuclear leukocytes (Fig. 9). In some places there is increased connective tissue extending within the lobules with slight infiltration with lymphocytes and polynuclear leukocytes. In these areas many of the liver cells are atrophic; elsewhere they appear normal.

The spleen shows marked hemorrhagic and necrotic areas. The adrenals show a perivascular exudate of lymphoid cells and moderate edema of the tissue throughout. The pancreas appears normal. Sections of the uterine tumor show an atrophic myoma.

The glomeruli of the kidneys are closely placed and large with considerable thickening of the capillary walls. There is an occasional completely sclerosed glomerulus. There is frequently an increased number of polynuclear cells in the capillaries. The interstitial tissue shows various stages from an acute to a healed process of uneven dis-

tribution. In many places there is an exudate chiefly of polynuclear cells extending in long streaks between the tubules (Fig. 10); in other places the exudate consists of lymphocytes and plasma cells, often situated about a glomerulus. In some places there is increased connective tissue. The tubular epithelium is granular, but shows little desquamation. The vessels show marked thickening of the intima and media with hyaline change. One section shows a small infarction.

Diagnosis.—Acute ulcerative endocarditis; subacute myocarditis; subacute interstitial hepatitis; subacute glomerular and interstitial nephritis; subacute perivascular exudate of adrenals; infarcts of spleen and kidney; congestion, hemorrhage, and edema of lungs; atrophic leiomyoma of uterus.

Bacteriological Data.—A blood culture was made on May 15, within 15 hours after the onset of systemic symptoms. 25 cc. of blood were drawn from the median basilic vein and distributed in dextrose agar and ascitic agar plates and deep tubes, plain and dextrose bouillon flasks and tubes, and Loeffler's blood serum slants. The cultures were incubated partly aerobically and partly anaerobically. On the third and fourth days after inoculation growth appeared in all the media, aerobic and anaerobic, except the dextrose agar plate and deep tube and the dextrose bouillon flask. These remained sterile. One colony appeared in the ascitic agar plate and three colonies in the ascitic agar deep tube. The colonies were grayish white, pin-head size, slightly irregular in outline. In bouillon the growth consisted of whitish flocculi which remained at the bottom of the tubes and flasks without clouding the media. In the Loeffler's blood serum slants the growth was most abundant, appearing as whitish flocculi in the blood and water of condensation at the bottom of the tubes.

A second blood culture was made on May 19. 20 cc. of blood were distributed in plain and ascitic bouillon, and Loeffler's blood serum tubes. No growth was observed in any of the media (seven days' incubation).

At autopsy, May 30, cultures of the heart's blood were made in 4 Loeffler's blood serum tubes and 3 bouillon flasks. After 48 hours' incubation all the blood serum tubes showed a moderate growth identical with that obtained in the first blood culture. One of the bouillon

flasks showed a staphylococcus which was considered a contaminant; the others remained sterile.

Cover-slip preparations from all the positive cultures showed a pure growth of a thin filamentous organism varying greatly in length. Many forms were homogeneous throughout their length, others fragmented giving the appearance of a chain of short bacilli. There were occasional forms containing deeply staining granules, and a few swollen spindle- and club-shaped forms. Here and there definitely branching forms were seen. These characteristics indicated that the organism was probably a streptothrix, a fact which further study has confirmed.

TABLE I.

Serum of patient E. S. A.	1:20	1:40	1:80	1:160	1:320	1:640	Salt solution.
2 hrs. at 37° C.	++	++	+	–	–	–	–
12 hrs. on ice	++	++	+	–	–	–	–
Normal human serum.	1:20	1:40	1:80	1:160	1:320	1:640	
2 hrs. at 37° C.	–	–	–	–	–	–	
12 hrs. on ice	–	–	–	–	–	–	

May 19. Numerous blood smears stained with Wright's stain and fresh dark-field preparations were carefully searched for parasites and organisms, but yielded negative results.

May 28. When the patient's temperature had fallen to normal, blood was drawn for agglutination tests with the organism isolated from the blood. 1 cc. of increasing dilutions of the patient's serum was mixed with 1 cc. of an 0.85 per cent salt solution suspension of the organism thoroughly shaken, in a series of small tubes and incubated for 2 hours at 37° C. in a water-bath. 0.85 per cent saline and the same dilutions of normal human serum were used as controls. Final readings were recorded after the tubes had stood in the ice-box for 12 hours. Positive agglutination occurred in dilutions of 1: 20, 1: 40, and 1: 80. The controls were negative. The results are shown in Table I (Fig. 7).

Agglutination tests were repeated in a similar manner after the

organism had been growing in subculture for three weeks, partial agglutination occurring in dilutions as high as 1:320 (Table II) after one hour's incubation.

TABLE II.

Serum of patient E. S. A.	1:20	1:40	1:80	1:160	1:320	1:640	Salt solution.
1 hr. at 37° C.	C	C	C	++	+	–	–
12 hrs. on ice.	C	C	C	++	+	–	–

C indicates complete agglutination and sedimentation.

Description of Organism.

Morphology.—The organism is a slender, filamentous, branching organism, growing in interwoven masses not manifesting a definite radial arrangement at the periphery of the colonies. In young cultures the filaments vary greatly in length, are curved, waving, or straight, show many true branching forms and stain homogeneously (Fig. 1). After 18 to 24 hours' incubation fragmentation of the filaments appears and branching forms rapidly disappear. The filaments now appear as chains of bacilli varying in length (Fig. 3). Staining is no longer homogeneous and numerous granular or beaded forms appear (Fig. 5). Occasional filaments become spindle-shaped, while others show ball-shaped, oval, or terminal club-shaped swellings, and chains of coccus-like forms appear (Fig. 2). Older cultures show marked fragmentation and breaking up of the filaments into rods (Fig. 4). The rods vary considerably in length, are straight or slightly curved, and are frequently beaded, resembling in many respects the pleomorphic bacilli. The chains of coccus-like forms become more numerous, and remaining masses of filamentous forms stain very faintly.

Staining Reactions.—The organism stains readily with methylene blue, gentian violet, carbol-fuchsin, pyronin, and Bismarck brown. It is Gram-negative and is neither acid- nor alcohol-fast.

Capsule.—No capsule formation could be demonstrated. The club-shaped forms do not show the characteristic sheath of actinomyces as described by Wright (24).

Spores.—No true endospores could be demonstrated.

Motility.—The organism is non-motile.

Oxygen Requirements.—The organism is a facultative anaerobe. Growth under anaerobic conditions is somewhat less abundant than under aerobic conditions.

Thermal Death Point.—The organism is killed by exposure to 60° C. for 10 minutes. Cultures containing chains of coccus-like forms were used in determining the thermal death point.

Viability.—Cultures kept at 37° C. die out after 5 to 8 days. On ice cultures remain viable 7 to 14 days.

Temperature Requirements.—The optimum temperature for growth is 37° C. Scanty growth appears after 4 days at room temperature.

Reaction of Media.—The optimum reaction for growth is between the neutral point and 2.0+. Growth does not occur in media of greater alkalinity than 1.5− or of greater acidity than 3.0+.

Pigment Formation.—The organism is non-chromogenic.

Cultural Characteristics.

Gelatin.—No growth.

Loeffler's Blood Serum.—After 24 hours at 37° C. a moderately abundant growth of discrete, whitish, pin-point, circular, sharply marginated, elevated, smooth, glistening, moist, non-mucoid colonies appears (Fig. 6). There is a whitish, flocculent growth in the water of condensation. The medium is not liquefied. After 48 hours' incubation colonies may show slight coalescence if closely aggregated, but they do not tend to increase in size after the first 24 hours' incubation.

Human Blood Agar.—After 24 hours at 37° C. a very scanty growth similar to that on Loeffler's blood serum appears.

Ascitic Agar.—When first isolated, the organism failed to grow on this medium. Growth was finally obtained, however, after the organism had been subcultured on Loeffler's blood serum for 2½ months. Colonies show the same characteristics as on blood serum.

Ascitic Bouillon.—(Plain bouillon 4 parts, ascitic fluid 1 part.) After 18 to 24 hours at 37° C. a whitish flocculent growth appears at the bottom of the tube and to some extent along the sides (Fig. 6).

The medium remains clear and there is no surface pellicle. Growth ceases after 24 to 30 hours.

Plain and dextrose agar, plain and dextrose bouillon, ox bile, litmus serum water, potato, and litmus milk showed no growth after 7 days' incubation on repeated trials.

Fermentation.—No growth could be obtained in levulose, lactose, dextrose, mannite, maltose, saccharose, or dextrin litmus serum waters. A good growth was obtained in inulin, lactose, mannite, raffinose, saccharose, and salicin broths to which ascitic fluid had been added, 1:4, but there was no demonstrable change in reaction after 7 days' incubation.

Pathogenicity.—Guinea pigs, rabbits, and white rats were inoculated with blood from the patient, and with varying amounts of 24 hour cultures of the organism with the following results.

Four rabbits were inoculated, two intravenously and two intraperitoneally, May 19, 1915, each with 5 cc. of citrated blood from the patient. One rabbit died on May 26, 1915, of an intercurrent infection. The other three showed no evidence of disease. Autopsies, June 30, 1915, were negative.

Two white rats were inoculated intraperitoneally, May 19, 1915, each with 3 cc. of citrated blood from the patient. Neither showed any evidence of disease. Autopsies, June 22, 1915, were negative.

Six guinea pigs were inoculated, May 20, 1915, three subcutaneously, three intraperitoneally, with varying amounts of ascitic bouillon cultures. None of the animals showed any evidence of disease. Autopsies, June 22 and 29, 1915, were negative.

Two rabbits were inoculated intravenously, two intraperitoneally, and two subcutaneously, May 20, 1915, with varying dosages of 24 hour ascitic bouillon cultures. Five yielded entirely negative results during 6 weeks' observation and at autopsy. The protocol of the sixth experiment is given below.

Rabbit 6.—Weight 1,220 gm. Inoculated subcutaneously, May 20, 1915, with a salt solution suspension of the growth from 30 cc. of ascitic bouillon (24 hours' incubation).

May 25. Animal seems well. A firm nodule the size of a large pea has appeared beneath the skin at the site of inoculation.

June 1. Nodule is the size of a small marble, firm, non-fluctuant, apparently not tender.

June 30. The animal has shown no other evidence of disease than the subcutaneous tumor which has persisted. There has been a steady gain in weight. Animal killed.

Autopsy.—Weight 1,590 gm. In the subcutaneous tissue of the left flank at

the site of inoculation is a firm, slightly irregular, encapsulated tumor 1.2 cm. in diameter. On section it presents a yellowish white granular appearance with two small foci of caseation. The lymph nodes in the left axilla are enlarged, discrete, and firm. On section they present a yellowish white, homogeneous surface without suppuration or caseation. No other enlarged nodes were noted. Heart, lungs, liver, spleen, kidneys, adrenals, gastro-intestinal tract, brain, and spinal cord appear normal macroscopically.

Microscopic Examination.—The subcutaneous nodule shows a peripheral wall of dense fibrous tissue surrounding a reticulum of loose connective tissue in which are many large and small necrotic foci surrounded by fibroblasts, plasma, and endothelial cells. No giant cells are seen. No organisms are demonstrable. The lymph nodes show only hyperplasia. Sections of the lung, myocardium, liver, spleen, and kidneys appear normal.

Cultures made from the subcutaneous nodule and lymph nodes remained sterile.

Four white rats were injected with varying amounts of 24 hour ascitic bouillon cultures with the following results.

White Rat 1.—May 22, 1915. Intraperitoneal injection of a salt solution suspension of the growth from 75 cc. of ascitic bouillon (24 hours' incubation). The animal appeared sick for a few days following the injection, but by the end of a week had apparently entirely recovered. It remained well until June 28, 1915, when it was killed.

Autopsy.—There were numerous fibrous adhesions about the spleen, stomach. liver, and diaphragm. The mesenteric, axillary, and inguinal lymph nodes were enlarged. They appeared glistening on section and showed no areas of caseation. No other abnormalities were noted on gross examination.

Microscopic Examination.—The lymph nodes showed only hyperplasia. Sections of the spleen showed thickening of the capsule and general hyperplasia. Plasma cells and endothelial cells were numerous throughout and occasional giant cells were seen. There were no areas of caseation or necrosis. The lungs, myocardium, liver, and kidneys appeared normal.

Cultures from the spleen, heart's blood, and lymph nodes remained sterile.

White Rat 2.—May 22, 1915. Intraperitoneal injection with a salt solution suspension of the growth from a 30 cc. ascitic bouillon culture.

May 23, 1915. Animal found dead. Streptococcus septicemia.

White Rat 3.—May 22, 1915. Subcutaneous injection with a salt solution suspension of the growth from a 30 cc. ascitic bouillon culture. The animal remained well without local or general reaction until June 13, 1915, on which date a small area of induration appeared at the site of inoculation. The lesion gradually increased in size, and on June 16, 1915, broke down, discharging a small amount of seropurulent fluid. The animal appeared lively and showed no evidence of a general reaction. The cutaneous lesion which was 1 cm. in diameter became crusted over but showed no tendency to heal or to spread. No other lesions developed. The rat was killed on June 28, 1915.

Autopsy.—At the site of inoculation there was a well localized abscess 1 cm. in diameter which had discharged externally and was covered with a reddish brown crust. The lesion was situated in the subcutaneous tissue and thoroughly walled off by dense connective tissue. The center was filled with necrotic material and a purulent exudate. The axillary, inguinal, peritoneal, and thoracic lymph nodes were considerably enlarged. On section the nodes appeared glistening grayish white, and showed no areas of caseation. No other gross abnormalities were noted.

Microscopic Examination.—The lymph nodes showed only hyperplasia and the presence of numerous plasma cells and endothelial leukocytes. Sections of the abscess showed a mass of necrotic material infiltrated at the margin with fibroblasts and plasma cells and surrounded by dense connective tissue. No giant cells were seen. Sections of the spleen, kidney, liver, lung, and myocardium appeared normal.

Smears from the abscess showed numerous rod-shaped and coccus-like organisms and a few filamentous forms. Cultures from the abscess showed an abundant growth in pure culture of the organism with which the rat was inoculated. Cultures of the heart's blood and lymph nodes remained sterile.

White Rat 4.—July 1, 1915. Subcutaneous injection of a salt solution suspension of the streptothrix isolated from Rat 3. The growth from 50 cc. of a 24 hour ascitic bouillon culture was used. The animal remained well and showed no local or general reaction. It was killed Aug. 11, 1915. Autopsy showed one enlarged axillary lymph node which on microscopic examination showed moderate hyperplasia. There were no other abnormalities.

In summary, the organism is very slightly pathogenic for rabbits and white rats, producing in some cases a local inflammatory and proliferative reaction at the site of inoculation, and general lymph node hyperplasia. It is not pathogenic for guinea pigs. Passage through one rat did not increase its pathogenicity.

DISCUSSION.

The diagnosis of rat-bite fever in the case described above seems thoroughly justified by the clinical picture which the patient presented. The history of a rat-bite, latent incubation period, non-suppurative lymphangitis, high fever ushered in by a severe chill, severe generalized muscular pain, stuporous condition, and the bluish red macular exanthem were quite typical of the recognized symptom-complex of the disease.

The isolation of an organism in pure culture from the blood during life and at autopsy and the development of an agglutinin in the

patient's blood serum for that organism leaves little question as to its causative relationship to the disease in this case. The presence of a morphologically similar organism in the sections of the mitral vegetation in the absence of other demonstrable organisms is strong presumptive evidence that the same organism was the cause of the endocarditis.

Study of the organism has shown it to be a true branching, filamentous organism, showing fragmentation of the mycelial filaments, granule formation, and the development of chains of coccus-like forms. It is Gram-negative and not acid-fast. In ascitic bouillon it grows as a flocculent sediment without clouding the media, and on solid media it grows in discrete colonies. These characteristics place the organism in the group of filamentous fungi, the Hyphomycetes. The nomenclature of both species and genera in this group is still somewhat confused, different authors using several generic names,—Nocardia, Actinomyces, Streptothrix, Discomyces, Oospora. The weight of opinion and common usage, however, favor the adoption of the name Streptothrix for the genus, and it seems best to classify the organism described above as one of the Streptothrices.

Claypole (25), Foulerton (26), and Musgrave, Clegg, and Polk (27) have done much to clarify our knowledge of this group of organisms. The latter authors give the following generic characters for the Streptothrices: "Branching, filamentous organisms which develop into colonies made up of the organisms and 'transformation products.' The terminal hyphæ may or may not be radially placed on the surface of the colony and they may or may not develop 'clubs.' The group in general take Gram's stain and several members show acid-fast properties in a varying degree." Claypole states that the members of the group are alike in the following characteristics: "When grown in bouillon, the medium always remains clear. The growth on solid media is discrete. . . . . They are all gram positive, all have granule formation, either in mycelium or its products, as shown in chain sporulation, and, finally, branching organisms and thread-like forms are seen more or less frequently in all species."

The organism isolated in this case shows all the typical features of a streptothrix with the exception that it is Gram-negative, a variation which does not seem sufficiently important to exclude it from the genus.

The question of species is a more difficult one. The literature contains numerous more or less complete descriptions of organisms isolated from cases of streptothricosis. Musgrave, Clegg, and Polk (27) recognize six well established species: *S. actinomyces*, Boestrom (1890); *S. actinomyces*, Wolff and Israel (1891), Wright (1905); *S. nocardii; S. eppingeri; S. maduræ*, Vincent; *S. capræ*, Silberschmidt. The organism isolated in our case is definitely distinct from any of these, and differs from any streptothrix hitherto described with the exception of that isolated by Schottmüller in his case of rat-bite fever. Although Schottmüller does not give a complete description of his organism, it agrees in most particulars with the organism isolated in our case, the only difference being that Schottmüller's organism was "tinged bluish" with Gram's stain and it did not grow at room temperature. In spite of these slight differences it seems probable that the organisms are identical, and for that reason advisable to accept his name for the organism,—*Streptothrix muris ratti.*

Two features of interest in our case in addition to the relation of the organism to rat-bite fever are isolation of a streptothrix in pure culture from the blood during life, and the presence of an ulcerative endocarditis caused by a streptothrix. Cases of generalized streptothricosis resembling pyemia are not infrequently recorded in the literature, but the only other instance of a streptothrix septicemia in which the organism was isolated by blood culture is Schottmüller's case of rat-bite fever. Cases of streptothrix endocarditis are equally rare. Naunyn (28) in 1888 found in endocarditis vegetations of a case of chorea a branching organism which was identified as a cladothrix or leptothrix by Zopf. Pappenheimer and Satchwell (29) isolated a filamentous organism from the blood in a case of endocarditis and demonstrated the same organism in sections of the aortic vegetations. They considered their organism to be a cladothrix. No other instances of endocarditis caused by a filamentous microorganism are recorded in the available literature.

SUMMARY.

The similarity in the cases of rat-bite fever recorded in the literature establishes it as a definite clinical entity. The same symptomatology occurs in cases from Asia, Europe, and America. The greater fre-

quency of the disease in Japan than elsewhere is probably due to the housing conditions and habits of the people resulting in the more frequent occurrence of rat-bites. It does not seem necessary to consider that cases occurring in Europe and America are due to the bites of rats that have been imported from Japan.

The clinical picture and course of the disease indicate that it is infectious in origin. Until Schottmüller's case appeared in 1914, the etiology had been undiscovered. He isolated from his case in eight consecutive blood cultures a streptothrix which he has designated *Streptothrix muris ratti*. His work has been confirmed by the isolation of an identical streptothrix from the blood during life and at autopsy in the case here reported. Further confirmation of the etiological relationship of this organism to the infection in our patient is found in the production of powerful agglutinins for the organism in the blood serum of this case and in the demonstration of the organism in the vegetation on the mitral valve. It is not unreasonable to suppose that Proescher (13) observed the same organism in the sections of the excised wound in his case. Although it is fully realized that Koch's postulates have not been fulfilled in the absence of successful animal experimentation, nevertheless the accumulated evidence here presented leaves little reason to doubt that the specific cause of rat-bite fever is *Streptothrix muris ratti*.

The pathology of rat-bite fever has hitherto been largely a matter of surmise. One autopsy only has been recorded in the literature (Miura (22)), and nothing abnormal was noted other than injection of the pial vessels. The autopsy in the case here reported has proved of considerable interest in the extent and character of the lesions found. A streptothrix septicemia with the localization of the organism in the mitral valve producing an acute ulcerative endocarditis is the most striking feature of the case. The infarcts of the spleen and kidney are a natural sequence of the endocarditis. The subacute lesions of the myocardium, liver, adrenal, and kidneys, glomerular and interstitial, are all of a similar nature, consisting of areas infiltrated with leukocytes, lymphocytes, plasma, and endothelial cells with varying degrees of degeneration of the normal cells of the affected area. In no instance has the presence of the streptothrix in these lesions been demonstrated, and it is not unreasonable to assume that they are toxic in origin.

The data here presented may be correlated with the clinical features of rat-bite fever to give us a clear understanding of the course and nature of the disease. The patient is inoculated by the bite of a rat with *Streptothrix muris ratti*. After a variable incubation period a non-suppurative inflammatory reaction occurs at the site of the wound with extension to the neighboring lymphatics and lymph nodes. Invasion of the blood stream follows, accompanied by the onset of severe toxic symptoms. Clinically the nervous system and frequently the kidneys seem to be especially involved. That the myocardium, liver, and adrenals may also suffer is shown by the autopsy findings in the case reported above. Ulcerative endocarditis is probably a rare occurrence. In the majority of cases after a more or less prolonged course, the disease terminates spontaneously and so may be considered a self-limited infection. This is presumably brought about by the development in the body of a protective mechanism against the streptothrix. That such a process does occur is evidenced by the demonstration of agglutinins in our case. Whether a permanent immunity is acquired after one attack of rat-bite fever is not known. No instances of a second infection are recorded in the literature.

Although rat-bite fever varies somewhat in its symptomatology in individual cases, the picture is sufficiently characteristic to make the diagnosis not a difficult matter. The history of a rat-bite, latent incubation period with subsequent non-suppurative inflammatory reaction of the wound, lymphangitis, and enlarged lymph nodes, severe chill at onset, high fever of the relapsing type, intense muscular pain and nervous symptoms, and the characteristic bluish red exanthem, present a symptom-complex not easily overlooked. The disease is frequently complicated by a severe nephritis, and prolonged cases develop a high grade of anemia and cachexia. In the case here reported ulcerative endocarditis occurred.

In the large majority of cases the prognosis is favorable for a successful termination. The patients, however, are often incapacitated for a considerable period of time The mortality is about 10 per cent, death usually occurring in the first febrile period apparently from a profound toxemia, or at a later stage due to the development of a severe nephritis.

Until recently treatment has been entirely symptomatic and has been of little avail in altering the course of the disease. Miyake has found immediate treatment of the wound by cauterization or with carbolic acid highly efficient as a prophylactic measure. Hata (30) in 1912 introduced salvarsan therapy and reported eight cases so treated, seven of which showed marked and rapid improvement. One case was apparently unaffected. Two of the cases receiving only small doses had a subsequent relapse. Surveyor (31) and Dalal (18) also have reported success with salvarsan injections. It is to be hoped that further experience with this method of treatment will yield equally favorable results.

CONCLUSIONS.

1. Rat-bite fever is a specific infectious disease following the bite of a rat. It occurs in Asia, Europe, and America.
2. The etiological organism is *Streptothrix muris ratti*, first described by Schottmüller in 1914. His observation is confirmed by the isolation of an identical streptothrix in the case here reported.
3. Invasion of the blood stream by *Streptothrix muris ratti* occurs in rat-bite fever.
4. The case here reported developed a powerful agglutinin for *Streptothrix muris ratti*.
5. Pathological changes occur in the myocardium, kidneys, liver, and adrenals showing areas of degeneration and infiltration with polynuclear leukocytes, lymphocytes, plasma cells, and endothelial cells.
6. Ulcerative endocarditis may occur in rat-bite fever and be caused by the *Streptothrix muris ratti*.

BIBLIOGRAPHY.

1. Miyake, H., *Mitt a.d. Grenzgeb. d. Med. u. Chir.*, 1900, v, 231.
2. Wilcox, W., *Am. Jour. Med. Sc.*, 1840, xxvi, 245.
3. Horder, T. J., *Quart. Jour. Med.*, 1909–10, iii, 121.
4. Millot-Carpentier, *L'union méd.*, 1884, xxxviii, 1069.
5. De Micas, *Arch. méd. de Toulouse*, 1895, i, 170.
6. Curtillet and Lombart, *Jour. de méd. int. Paris*, 1912, xvi, 335.
7. Peña y Maya, F., *Siglo Med.*, 1885, xxxii, 117.
8. Frugoni, C., *Riforma med.*, 1911, xxvii, 1298.

9. Schottmüller, H., *Dermat. Wchnschr.*, 1914, lviii, Supplement, 77.
10. Ogata, *Mitt. a. d. med. Fakult. d. k. Univ. z. Tokyo*, 1909, viii, 287.
11. Atkinson, A. G., *Med. Chron., Manchester*, 1912–13, series 4, lvii, 1.
12. Gouget, A., *Presse méd.*, 1912, xx, 186.
13. Proescher, F., *Internat. Clin.*, 1911, series 21, iv, 77.
14. Crohn, B. B., *Arch. Int. Med.*, 1915, xv, 1014.
15. Farquhar, O. C., *Cincinnati Lancet and Obs.*, 1869, xii, 650.
16. Nixon, J. H., *Brit. Med. Jour.*, 1914, ii, 629.
17. Dick, M. I., and Rutherford, W. J., *Brit. Med. Jour.*, 1913, ii, 1581.
18. Dalal, A. K., *Practitioner*, 1914, xcii, 449.
19. Ogata, M., *Mitt. a. d. med. Fakult. d. k. Univ. z. Tokyo*, 1911, ix, 343.
20. Ogata, *Mitt. a. d. med. Fakult. d. k. Univ. z. Tokyo*, 1913–14, xi, 179.
21. Middleton, G. S., *Lancet*, 1910, i, 1618.
22. Miura, M., and Toriyama, N., *Ztschr. d. med. Gesellsch. z. Tokyo*, 1897, xi, 1059.
23. Reece, M., *Med. Exam.*, 1872, xiii, 51.
24. Wright, J. H., *Jour. Med. Research*, 1904–05, xiii, 349.
25. Claypole, E. J., *Jour. Exper. Med.*, 1913, xvii, 99.
26. Foulerton, A. G. R., *Lancet*, 1910, i, 552, 626, 769; 1913, i, 381.
27. Musgrave, W. E., Clegg, M. T., and Polk, M., *Philippine Jour. Sc.*, 1908, iii, B, 447.
28. Naunyn, B., Mittheilungen aus der medizinischen Klinik zu Königsberg i. Pr., Leipzig, 1888, 296.
29. Pappenheimer, A. M., and Satchwell, H. H., *Jour. Infect. Dis.*, 1907, iv, 617.
30. Hata, S., *München med. Wchnschr.*, 1912, lix, 854.
31. Surveyor, N. F., *Lancet*, 1913, ii, 1764.

EXPLANATION OF PLATES.

Plate 8.

Fig. 1. Stained preparation of *Streptothrix muris ratti* from a 15 hour Loeffler's blood serum culture, showing branching, homogeneously stained filaments and the non-radial arrangement at the margin of the colony.

Fig. 2. *Streptothrix muris ratti* from a 24 hour Loeffler's blood serum culture, showing irregularly swollen filaments and beginning formation of coccus-like chains.

Plate 9.

Fig. 3. Fragmentation of mycelial threads appearing as chains of bacilli (24 hour culture).

Fig. 4. Complete fragmentation into rod-like forms (30 hour culture).

Fig. 5. Fragmented forms showing beading and terminal swellings.

PLATE 10.

FIG. 6. Twenty-four hour culture of *Streptothrix muris ratti* on Loeffler's blood serum and in ascitic bouillon.

FIG. 7. Agglutination of *Streptothrix muris ratti* by patient's serum. Dilutions 1 : 20, 1 : 40, 1 : 80, and control (Table I).

PLATE 11.

FIG. 8. Section of myocardium from patient.

FIG. 9. Section of liver from patient.

PLATE 12.

FIG. 10. Section of kidney from patient.

PLATE 13.

FIG. 11. Section of mitral vegetation showing necrotic material, infiltration with polymorphonuclear leukocytes, and masses of organisms.

PLATE 14.

FIG. 12. Section of mitral vegetation showing clumps of organisms. The rod-like form of the organisms is indefinite in the single focal plane represented in the microphotograph, but can easily be demonstrated in microscopic study of the actual section.

ANTIBLASTIC IMMUNITY.

By A. R. DOCHEZ, M.D., and O. T. AVERY, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 19, 1915.)

As a result of the study of the natural or acquired resistance of animals against infection with living microorganisms, many previously unknown properties of the cells and fluids of the body have been discovered. The value of these properties as resistance factors is dependent upon the degree of antagonism which they manifest to invasion with living foreign elements. On the other hand, virulence of bacteria may be said to be proportional to the strength of defence which they possess against such antagonistic forces. In the face of such opposition, however, the bacterium must not only maintain its life, but in order to increase must be able to obtain a proper food supply. The mechanism by means of which bacteria extract from living tissue the substances essential to their nutrition is not well understood, nor can we say to what extent the animal body opposes this appropriation of its own supplies. It is in the hope of throwing light upon this obscure problem that the following study has been undertaken.

The microorganism used was the pneumococcus, not because it is especially suitable, but because many of the materials necessary for the experiments were easily obtainable. The hypothesis upon which our efforts are based is that bacteria do not assimilate all of their foodstuffs in the condition in which they exist in the medium of their environment, but that certain changes must be effected before absorption occurs. The preparation of nutritional substances may take place upon the surface of the bacterial cell or in its immediate neighborhood, and this function, when carried on within the substance of a living animal, may be opposed by certain inhibitory forces. The interaction of these phenomena may play an important part in resistance and immunity to infection.

By immunization of the horse to pneumococcus, a serum can be prepared which protects susceptible animals against many times the fatal dose of virulent pneumococci. A complete understanding of the mode of action of this serum is still lacking. However, the fact has been observed and confirmed that antipneumococcus serum possesses neither bactericidal nor bacteriolytic action *in vitro*. Indeed, pneumococcus is known to grow in considerable concentration of its homologous immune serum. In the following experiment (Table I) is shown the rate of growth of pneumoccoccus in homologous and in heterologous antipneumococcus serum and in normal horse serum. The amount of growth was determined by the plate method.

TABLE I.

Experiment I. Inhibition of Growth of Pneumococcus by Antipneumococcus Serum.

Serum 0.1 cc.	Culture 0.00001 cc.	Immediately.	After 3 hrs.
Antipn. serum Type I...........	Pn. I	230 colonies.	216 colonies.
" " " II...........	" I	200 "	630 "
Normal.........................	" I	200 "	1,400 "

This experiment shows clearly that a marked inhibition of the growth of pneumococcus occurs in antipneumococcus serum, as compared with normal horse serum. The inhibitory effect of homologous immune serum is greater than that of heterologous immune serum; in fact, in the former serum no increase in the number of pneumococci has taken place in three hours. The inhibition of growth may be explained in four ways: first, that agglutination occurs in immune serum, and that the failure to increase is apparent only; second, that the formation of threads is responsible for the apparent inhibition; third, that we are dealing with the bacterial lag of freshly planted cultures; and fourth, that active interference with the growth phenomena of the organisms has occurred. That the first two reasons are entirely responsible for the inhibition of growth is disproven by the fact that a marked delay in development occurs in heterologous immune serum in which no agglutination whatever takes place and in which thread formation is not more extensive than in normal serum. The latent period that marks the growth of freshly planted

cultures of bacteria cannot explain the inhibition, inasmuch as this would affect the culture in normal serum as well as that in immune serum, since the same culture of pneumococcus was used for seeding and the conditions of cultivation were similar in each tube. We are forced, then, to conclude that these various phenomena do not entirely explain the inhibition of growth, and that such inhibition as occurs is largely dependent upon some property of immune serum which adversely affects the circumstances of multiplication. The inhibitory influence of immune serum is manifest only for a relatively short period of time, for if plates are made at the end of twenty-four hours, the pneumococcus is found to have overcome the inhibition, and innumerable colonies develop from comparable amounts of all the cultures.

Carbohydrate and protein form the main food supply of most bacteria. In our hypothesis we have suggested that these substances may not be absorbed unchanged and that some form of preparation may occur at the surface of the bacterial cell. If immune serum is added to the medium in which the cell is growing, the changes necessary for the development of food substances appropriate for assimilation would be subject to the influence of any inhibitory bodies present in the immune serum. In the following experiments evidence is brought that antipneumococcus serum possesses the power to inhibit both the splitting of protein and the fermentation of sugar by pneumococcus. The organisms were grown in serum broth for twenty-four hours. The amount of protein splitting has been estimated by the increase in amino nitrogen determined by the method of Van Slyke.

The following experiment is one of a series showing the degree of protein splitting that occurs when pneumococci are grown in broth containing normal horse serum, and the effect of substituting antipneumococcus serum for normal serum in the mixtures. The estimations of the increase in amino nitrogen were made after twenty-four hours' incubation at 37° C., a period at which marked growth of the pneumococcus had occurred both in the tubes containing normal serum, and in those containing immune serum. In those tubes in which the pneumococcus has grown in its homologous serum, agglutination has occurred. There has been, however, no agglutination either in normal serum or in heterologous immune serum.

TABLE II.

Experiment II. Inhibition of Digestion of Protein by Pneumococcus with Antipneumococcus Serum.

	Increase in amino nitrogen per cc.
Pn. Type I + 1 cc. normal horse serum + 4 cc. broth..........	0.21 cc. nitrogen.
" " I " 1 " antipn. serum Type I + 4 cc. broth.......	0.02 " "
" " I " 1 " " " " II " 4 " "	0.06 " "
" " II " 1 " normal horse serum " 4 " "	0.21 " "
" " II " 1 " antipn. serum Type I " 4 " "	0.14 " "
" " II " 1 " " " " II " 4 " "	0.06 " "

The figures of this experiment (Table II) make it strikingly evident that the addition of antipneumococcus serum to growing cultures of pneumococcus markedly diminishes, in some instances almost to the point of extinction, the production of amino-acids by the organism. The formation of amino-acids in mixtures of broth and normal horse serum has been found generally equivalent to the amount found in broth alone. Certain other normal animal sera have, however, been observed which inhibit the process in varying degrees. Inhibition by homologous immune serum is most complete, although, as in the experiment showing inhibition of growth of pneumococcus, heterologous immune serum possesses considerable inhibitory power. Whether the increase in amino-acid is due to endogenous or exogenous metabolism cannot, of course, be definitely determined. We believe, however, that, in the utilization of protein, the pneumococcus effects a splitting of the protein before absorption, and that the increase in amino-acid represents the excess of protein split, over that used up in the process of growth.

Carbohydrate, as is well known, is an important part of the food supply of most bacteria, and the great variety of sugars that many organisms ferment is extraordinary. That such a splitting of sugars is in some way associated with the nutrition of microorganisms seems a logical assumption. We have found that the addition of antipneumococcus serum to cultures of pneumococcus containing such sugars as glucose, saccharose, lactose, and inulin inhibits in varying degrees the fermentation of the sugars by the organism. Inhibition of fermentation of inulin is most complete, and an example of this inhibition is given in Experiment III (Table III). Pneumococcus, when added to serum water media containing inulin, and litmus as

an indicator, ferments the inulin which results in acidification of the medium and coagulation of the serum.

TABLE III.

Experiment III.

	24 hrs.	48 hrs.	72 hrs.	5 days.
Pn. Type I + inulin	++	++	++	++
" " I " antipn. serum Type I + inulin.	–	–	–	–
" " I " " " " II " "	Sl. ac.	Ac.	++	++

++ indicates complete acidification and coagulation; + indicates acid and incomplete coagulation; ± indicates acid and beginning coagulation; V. sl. ac. indicates very slight acidification; Sl. ac. indicates slight acidification; Ac. indicates slight acidification and no coagulation; – indicates no acidification or coagulation.

Experiment III shows that the addition of homologous immune serum to a culture of pneumococcus in inulin completely suspends fermentation of the inulin. Heterologous immune serum delays the reaction, but does not entirely inhibit it. Fermentation of the sugars more actively attacked by pneumococcus, such as glucose, lactose, and saccharose, is not inhibited to the same degree as that of inulin. Determination of the rate of production of acid in cultures containing such easily fermentable sugars shows that, in the early hours of growth, the formation of acid is markedly delayed by the presence of immune serum. After twenty-four hours, however, the acid concentration may reach the same degree in all tubes and, in general, represents the grade of acidity at which pneumococcus ceases to grow. The splitting of carbohydrates by bacteria probably occurs at the surface of the bacterial cell, as is thought to be the case in fermentation of sugar by yeast, and the anti-enzymotic forces of immune serum in all probability exert their antagonistic action at this point.

A study of human blood serum obtained at intervals during the course of an attack of lobar pneumonia shows that bodies having an anti-enzymotic action similar to that of immune serum develop during the period of recovery from the disease. The tests were made in the same manner as those in which an artificially prepared immune

serum was used. The two following experiments are typical of the results obtained (Tables IV and V).

TABLE IV.

Experiment IV. Inhibition of Digestion of Protein by Pneumococcus with Human Serum in Lobar Pneumonia. Infection with Pneumococcus Type II.

	Increase in amino nitrogen per cc.
Pn. Type II + 2 cc. broth + 0.5 cc. serum, 5 days before crisis.....	0.16 cc. nitrogen.
" " II " 2 " " " 0.5 " " at crisis................	0.02 " "
" " II " 2 " " " 0.5 " " 9 days after crisis......	0.02 " "

TABLE V.

Experiment V. Inhibition of Fermentation of Inulin by Pneumococcus with Human Serum in Lobar Pneumonia. Infection with Pneumococcus Type I.

	24 hrs.	48 hrs.	72 hrs.	5 days.
Inulin + Pn. Type I..............................	++	++	++	++
" " " " I + serum 8 days before crisis...	±	++	++	++
" " " " I " " 6 " " " ...	+	++	++	++
" " " " I " " just after crisis.......	−	V.sl.ac.	Sl.ac.	Ac.
" " " " I " " 7 days " "	Sl.ac.	Ac.	±	±

These experiments show clearly that at the crisis of lobar pneumonia substances appear in the serum either for the first time, or in greatly increased amount, which have the power of inhibiting the proteolytic and glycolytic activities of the pneumococcus. The period of development of these substances corresponds in time with that of other immune bodies which have been recognized in the serum of individuals with lobar pneumonia.

DISCUSSION.

The series of experiments presented in this paper demonstrate the following facts. Antipneumococcus serum possesses the power of inhibiting for a certain period of time the multiplication of pneumococci. In conjunction with this capacity, it has also the power of inhibiting in varying degree the proteolytic and glycolytic functions of pneumococci. This power is present to a limited extent in the

sera of certain normal animals and absent in others, and in human sera during the course of an attack of lobar pneumonia it appears for the first time or increases markedly at the critical period of the disease. From these facts we are led to assume that retardation of growth is, in part at least, dependent upon inhibition of metabolic function. The observation that immune serum possesses in high degree the powers described, suggests that these properties play an important part in resistance and immunity to infection with pneumococcus. Investigators have demonstrated previously that certain other immune sera possess analogous qualities; such as the inhibition of pigment production by *Bacillus pyocyaneus* (1), the liquefaction of gelatin by *Staphylococcus pyogenes aureus* (2), and the formation of methemoglobin by pneumococcus (3). We have chosen the term "antiblastic immunity" as descriptive of this phenomenon, in order to indicate that the forces at work are antagonistic to the growth activities of the organism. Ascoli (4) coined the term several years ago (*βλαστειν*, to grow). From his studies in anthrax immunity, he was led to suppose that the latter was in part dependent upon the inhibition of formation by *Bacillus anthracis* of a capsule which is a prerequisite for its successful development in the animal body, and he ascribed to anti-anthrax serum an antiblastic action, directed against the metabolic activity of this organism. A concrete interpretation of this phenomenon as applied to the growth of pneumococcus and the inhibitory influence of immune serum is as follows: Pneumococcus, in order to grow, must obtain a sufficient supply of protein and carbohydrate; these substances are furnished by the environmental medium, but probably require, to render them suitable for absorption, preliminary preparation in the nature of digestion. This change is effected at the surface of the bacterial cell and the integrity of this digestive zone is essential to the growth of the bacterium. Anti-enzymotic bodies such as have been demonstrated in immune serum act at the point of contact of the cell with its environment, and influence in an unfavorable manner the nutritional processes there carried on, and the consequence of such action is retardation or inhibition of growth. It is possible that capsule formation represents on the part of the organism an attempt to protect the function of the digestive zone. Should the foregoing prove

to be a correct explanation of the phenomenon observed, considerable light would be thrown on the obscure mechanism by means of which parasitic bacteria establish themselves in animal tissues, and on the forces mobilized by the animal body in opposition to such invasion.

CONCLUSIONS.

1. Antipneumococcus serum possesses the power of inhibiting for a certain period of time the multiplication of pneumococci.
2. It also has the capacity of inhibiting the proteolytic and glycolyt˙c functions of pneumococci.
3. This power is acquired for the first time or appears in increased amounts in human serum at the time of crisis in lobar pneumonia.
4. The retardation of bacterial growth is thought to be dependent upon the inhibition of metabolic function due to the presence of anti-enzymotic substances in antipneumococcus serum. To this phenomenon we have applied the term antiblastic immunity.

BIBLIOGRAPHY.

1. Ghéorghiewsky, *Ann. de l' Inst. Pasteur*, 1899, xiii, 298.
2. von Dungern, *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1898, xxiv, 710.
3. Cole, R., personal communication.
4. Ascoli, A., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1908, xlvi, 178.

ATHEROMA AND OTHER LESIONS PRODUCED IN RABBITS BY CHOLESTEROL FEEDING.

By C. H. BAILEY, M.D.

(*From the Pathological Laboratory of the Medical School of Leland Stanford Junior University, San Francisco.*)

Plates 15 to 19.

(Received for publication, July 8, 1915.)

Ignatovski (1) in 1908 published the results of his experiments on the effect of animal food on the rabbit. By feeding milk and egg yolk to these animals he produced various lesions, the most noteworthy of which were atheroma of the aorta, cirrhosis of the liver, and enlargement of the adrenals. In the following year Starokadomsky and Ssobolew (2), also by feeding rabbits on milk and egg, confirmed the results of Ignatovski in so far as the production of an atheroma of the aorta was concerned. They also obtained lesions in the innominate artery, carotids, and subclavians. Ignatovski ascribed his results to animal proteids. Several workers had previously described lesions produced in rabbits by animal food (d'Amato (3) lesions of the aorta, and Garnier and Simon (4) lesions of the liver), but these lesions differed essentially from those produced by Ignatovski and the latter's results attracted particular attention on account of the similarity of the aortic lesions as described by him to human atheroma. Stuckey (5) also confirmed Ignatovski's results. He afterward attempted to determine what part of the food given was responsible for the lesions, trying (6) milk, meat juice, egg albumin, and egg yolk, with the result that lesions as described by Ignatovski were produced only by the egg yolk. Later (7) he used different animal and plant fats, fish liver oil, beef fat, and sunflower-seed oil with negative results. He did, however, produce lesions of the aorta, apparently identical with those described by Ignatovski, by feeding rabbits on brain.

Chalatow (8) studied the livers of Stuckey's rabbits and reported characteristic changes in the livers of those which had been fed on egg and brain. In these he found an abundant infiltration of the parenchyma with doubly refracting fat.

Wesselkin (9) fed rabbits on egg and milk and others on lecithin and milk. In the former he obtained an abundant deposit in the liver, aorta, and spleen of lipoid substances which he considered mainly cholesterol esters. In the lecithin-fed rabbits also he found in the same organs a deposit of lipoids which, from their microchemical reactions, he believed to be phosphatids. Because, however, no pathological changes followed the deposit of the latter in the organs, while marked changes were produced by the cholesterol esters, he concluded that the injury was to be ascribed to the cholesterol of the egg yolk.

Anitschkow and Chalatow (10) fed pure cholesterol and produced characteristic lesions, and later also Wacker and Hueck (11). Weltmann and Biach (12), however, though they produced a cirrhosis of the liver, were unsuccessful in obtaining aortic lesions; and van Leersum (13) who fed two rabbits on eggs and two on pure cholesterol found a suspicious lesion in but one of the four. This was an egg-fed rabbit which had been under treatment for a shorter period than any of the other three. These authors are unable to explain their lack of results. Van Leersum states that spontaneous atheroma occurs in about 3 per cent of rabbits, and apparently believes the one lesion observed in his rabbits is to be so explained.

Anitschkow and Chalatow (10, 14) produced similar lesions in guinea pigs by egg feeding, with an abundant deposit of anisotropic fat in the organs, but failed entirely in rats. Adler (15) found aortic lesions in each of two dogs fed on cholesterol, and he also found lesions in two of three dogs receiving cottonseed oil.

Aschoff (16) and Anitschkow (17) believe that other factors, especially an increase in blood pressure, are important in the production of the aortic lesions. The latter believes that the formation of such lesions in these rabbits is facilitated by increasing the blood pressure in the aorta by constricting its lumen in the abdominal portion, by suspending the animals according to the method of Klotz (18), or by the use of adrenalin.

For the purpose of studying the lesions produced by cholesterol-containing food, nine rabbits were fed on egg yolk and six on pure cholesterol. The egg yolk and the cholesterol, the latter dissolved in cottonseed oil by heating, usually in the proportion of 0.5 gm. to 10 cc. of oil, were mixed with the rabbits' daily ration of crushed barley. On an occasional day greens were given with the barley without the addition of egg or cholesterol. One rabbit, as a control, received large doses of cottonseed oil without cholesterol. This rabbit was fed on oil over a much longer period than any of the rabbits receiving cholesterol, and none of the latter received as much oil in the course of their feeding as did the control in a similar period.

Smears and frozen sections of the fresh tissue were examined for doubly refracting fat under the polarizing microscope. Frozen sections of the tissues fixed in Orth's fluid were stained with Sudan III and by Van Gieson's method; paraffin sections with Van Gieson's method, hematoxylin and eosin, and in some cases with Weigert's elastic tissue stain and Mallory's connective tissue stain.

EXPERIMENTAL.

Rabbit 1.—Control. Received 1,216 cc. of cottonseed oil in 248 days. For short periods no oil was given; the remainder of the time it took from 3 to 23 cc. of oil per day. At autopsy no gross lesions were found. The left adrenal was larger than normal, the right very small, measuring but 2 mm. in diameter. The combined weight was normal,—0.28 gm. On microscopical examination the aorta, liver, spleen, and kidneys were found normal. The liver contained a very small amount of isotropic fat in the peripheries of the lobules. No anisotropic fat was found except in the adrenals.

Rabbit 2.—Received 0.01 gm. of cholesterol per day with the exception of an occasional day. Took 0.29 gm. of cholesterol in 33 days.

Gross Appearances.—Aorta, pulmonary artery, spleen, liver, and kidneys normal. Adrenals normal size; combined weight, 0.37 gm.

No anisotropic fat was found in the aorta, spleen, liver, or kidneys.

Rabbit 3.—Received 0.01 gm. of cholesterol per day with the exception of an occasional day. Took 0.5 gm. in 55 days.

Gross Appearances.—Aorta, pulmonary artery, spleen, liver, and kidneys normal. Adrenals normal size; combined weight, 0.35 gm.

No anisotropic fat was found in the aorta, liver, spleen, or kidneys.

Rabbit 4.—Received 0.05 gm. of cholesterol per day with the exception of an occasional day. Took 3.8 gm. in 84 days.

Gross Appearances.—Aorta, pulmonary artery, spleen, liver, and kidneys normal. Adrenals slightly enlarged; combined weight, 0.41 gm.

No anisotropic fat was found in the aorta, liver, or kidneys; a very small amount in the spleen.

Rabbit 5.—Received 1 egg yolk per day with the exception of an occasional period of a few days. Took 49 yolks in 78 days.

Gross Appearances.—Pronounced intimal lesions in aorta and pulmonary artery. Spleen about three times normal size. Liver slightly enlarged, surface smooth, cuts with somewhat increased resistance, shows pronounced fine deep yellow mottling. Adrenals greatly enlarged; combined weight, 1.35 gm. Kidneys show a few small scars on the surface; in the medulla, mainly in the outer zone, but extending into the inner zone are very numerous yellow opaque streaks and nodules. Large amount of anisotropic fat in the aortic lesions, liver, spleen, and kidneys.

Rabbit 6.—Received from 0.1 gm. to 0.5 gm. of cholesterol per day with the exception of a few days. Took 16.4 gm. of cholesterol in 83 days.

Gross Appearances.—Moderate intimal lesions in the aorta, confined to arch and thoracic portion, and in pulmonary artery. Spleen about normal size. Liver normal size, surface smooth, cuts with somewhat increased resistance, shows pronounced fine yellow mottling. Adrenals slightly enlarged; combined weight, 0.4 gm. Kidneys show a few small scars on the surface, a few opaque yellow lesions in the outer zone of medulla. Large amount of anisotropic fat in the aortic lesions, liver, spleen, and kidneys.

Rabbit 7.—Received 1 egg yolk per day with the exception of an occasional period of a few days. Took 58 yolks in 85 days.

Gross Appearances.—Surface of aorta dull, but no definite lesions apparent here or in the pulmonary artery. Spleen normal. Liver slightly enlarged and yellow. Adrenals enlarged; combined weight, 0.69 gm. Kidneys show no scars on surface, a few very faint yellow lines in outer zone of medulla. Moderate amount of anisotropic fat in intima of aorta, large amount in liver, very small amount in spleen and kidneys.

Rabbit 8.—Received 1 egg yolk per day with the exception of an occasional day. Took 26 yolks in 30 days.

Gross Appearances.—Pronounced intimal lesions in aorta, most numerous in arch and thoracic portion, but present throughout, also in pulmonary artery. Spleen normal in size. Liver shows pronounced fine yellow mottling, otherwise normal. Adrenals slightly enlarged (not weighed). Kidneys show no scars on surface; medulla is a diffuse yellowish color with a few definite yellow streaks in the outer zone.

Anisotropic fat in aortic lesions, liver, spleen, and kidneys.

Rabbit 9.—Received 1 egg yolk per day with the exception of an occasional period of a few days. Took 47 yolks in 98 days.

Gross Appearances.—Very marked intimal lesions throughout aorta, extending into large vessels of neck, renal arteries, and iliacs; also in pulmonary artery. Spleen moderately enlarged. Liver appears normal. Adrenals enlarged; combined weight, 0.56 gm. Kidney shows numerous scars on surface, and in outer zone of medulla very thickly set and pronounced yellow nodular lesions, extending as fine yellow lines into inner zone.

Large amount of anisotropic fat in aortic lesions and kidney, moderate amount in liver and spleen.

Rabbit 10.—Received 0.5 gm. of cholesterol per day with the exception of an occasional day. Took 14.5 gm. in 36 days.

Gross Appearances.—Moderate intimal lesions in aorta, mostly in arch and thoracic portion, a few in abdominal portion; also in pulmonary artery. Spleen moderately enlarged. Liver shows pronounced fine yellow mottling. Adrenals slightly enlarged (not weighed). Kidneys show a few small scars on the surface, numerous yellow lesions in outer zone of medulla extending into inner zone. Large amount of anisotropic fat in aortic lesions, liver, spleen, and kidneys.

Rabbit 11.—Received 1 egg yolk per day with the exception of an occasional interval of a few days. Took 54 yolks in 103 days.

Gross Appearances.—Very marked intimal lesions throughout aorta, most marked in arch and thoracic portion, also in iliacs; marked lesions in pulmonary artery. Spleen moderately enlarged. Liver shows pronounced yellow mottling. Adrenals enlarged; combined weight, 0.58 gm. Kidneys show numerous scars on surface; in outer zone of medulla numerous very pronounced yellow lesions, extending also into inner zone. Large amount of anisotropic fat in aortic lesions, liver, and kidneys; small amount in spleen.

Rabbit 12.—Received $\frac{1}{2}$ to 1 egg yolk per day with the exception of an occasional interval. Took 68 yolks in 138 days.

Gross Appearances.—Marked lesions in the arch of the aorta, smaller lesions in the thoracic and abdominal portions; moderate lesions in the pulmonary artery. Spleen moderately enlarged. Liver about normal size, surface smooth, cuts with somewhat increased resistance, shows pronounced yellow mottling. Adrenals enlarged; combined weight, 0.72 gm. Kidneys appear normal. Large amount of anisotropic fat in aortic lesions and liver, a moderate amount in spleen, very little in kidneys.

Rabbit 13.—Received 1 egg yolk per day with the exception of an occasional day. Took 27 yolks in 34 days.

Gross Appearances.—Moderate intimal lesions in aorta and pulmonary artery. Spleen about normal size. Liver appears normal. Adrenals enlarged; combined weight, 0.55 gm. Kidneys show numerous irregular depressed scars on surface, in outer zone of medulla a moderate number of yellow opaque streaks. Anisotropic fat abundant in aortic lesions and kidney, small amount in liver and spleen.

Rabbit 14.—Received 0.1 to 0.3 gm. of cholesterol per day with the exception of an occasional interval of a few days. Took 8.6 gm. of cholesterol in 97 days.

Gross Appearances.—Very pronounced intimal lesions from valves to exits of renal arteries, a few small lesions below; marked lesions in pulmonary artery. Spleen normal size. Liver shows slight yellow mottling, otherwise normal. Adrenals greatly enlarged; combined weight, 1.4 gm. Kidneys show no scars on surface, in outer zone of medulla a slight diffuse yellow color. Large amount of anisotropic fat in aortic lesions and liver, moderate amount in kidney, very little in spleen.

Rabbit 15.—Received 1 egg yolk per day with the exception of an occasional day. Took 65 yolks in 79 days.

Gross Appearances.—Marked intimal lesions in aorta and pulmonary artery. Spleen about twice normal size. Liver shows pronounced yellow mottling, otherwise normal. Adrenals enlarged, combined weight, 0.84 gm. Kidneys appear normal. Large amount of anisotropic fat in aortic lesions, liver, and spleen; only a very few fine droplets in kidney.

Rabbit 16.—Received $\frac{1}{4}$ to $\frac{1}{2}$ egg yolk per day with the exception of an occasional interval of a few days. Took $32\frac{1}{2}$ yolks in 134 days.

Gross Appearances.—Slight intimal lesions in arch and upper thoracic portion of aorta, none elsewhere; slight lesions in pulmonary artery. Spleen moderately enlarged. Liver shows slight fine yellow mottling. Adrenals enlarged; combined weight, 0.62 gm. Kidneys appear normal. Large amount of anisotropic fat in aortic lesions and liver, a moderate amount in the kidney, little in the spleen.

The previously mentioned authors agree in the main in their descriptions of the aortic lesions. To summarize very briefly, they describe the production of yellowish white raised plaques and stripes

mainly in the arch of the aorta. Microscopically these lesions were mainly intimal and showed a pronounced thickening due to a collection of cells, prominent among which were very large cells filled with doubly refracting fat. Similar fat was also found lying outside the cells. In the later lesions considerable production of elastic fibers was observed with some degeneration of the large cells. A small amount of doubly refracting fat was present in the upper layers of the media. The adventitia was not affected.

The preceding brief summary of our experiments shows that in eleven of the fifteen rabbits lesions of both aorta and pulmonary artery were visible in the gross. One additional rabbit, No. 7, in which lesions were not evident macroscopically, showed very numerous characteristic but relatively slight lesions microscopically. The gross lesions varied greatly in number, size, and severity, but were always quite evident (Fig. 1). They were invariably most marked in the arch and thoracic portion and in some of the less severe cases were found only here. In several of the latter the lesions were especially prominent about the exits of the large vessels arising from the arch or of the intercostals; in others a tendency to this localization was not apparent. The lesions consisted of small (in the early cases a fraction of a mm.) raised, yellowish white, rather sharply defined spots, or less frequently of fine streaks lengthwise of the vessel. In more marked cases larger irregular areas, several mm. in diameter, were seen evidently formed by the confluence of many smaller spots. These in cases involved a large portion of the arch and thoracic portion, leaving only comparatively small irregular areas of normal intima. The lesions of the pulmonary artery were similar to those of the aorta, but in most cases not so pronounced.

The intima of the rabbit's aorta is normally very thin, consisting of a single layer of endothelium and possibly an occasional fibroblast lying on the internal elastic layer of the media. The early lesions produced by cholesterol feeding consist of a cellular thickening of the intima, most of the cells present showing small droplets of doubly refracting fat. This fat is seen in spindle cells, a few drops appearing at either end of the nucleus, and in large, swollen, round or somewhat irregular cells which are loaded with the fat. The nuclei of these

cells vary, some being small, round, and deeply staining, others larger, round or oval, and vesicular.

There has been considerable discussion as to the nature of these fat-containing cells, as there has been concerning the similar cells seen in human atheroma. Large cells similar in appearance and loaded with doubly refracting fat are seen in various localities in these rabbits, in the bone marrow, in the spleen both in the pulp and free in the venous sinuses, and in the kidney in the capillaries of the medulla and in interstitial lesions to be described. These large fat-containing cells in the situations mentioned are apparently of endothelial type (histiocytes of Kiyono), and it would seem probable that the similar cells in the aorta are of the same nature. In marked cases, however, the fat is also seen in cells which are evidently fibroblasts, as in the epicardial connective tissue, about the bronchi in the lungs, and in the capsule and trabeculæ of the spleen. In these cells the fat is present as a few small droplets at either end of the nucleus. The fat content of the cells in these situations is always small and the large swollen cells are not seen. It is believed that in the intimal lesions of the aorta also fibroblasts take some part in the absorption of the fat.

Anitschkow (19) calls attention to the similarity in the distribution of the doubly refracting fat to that of the dye in vital staining. In the latter process also fibroblasts show a few granules of the dye at either end of the nucleus, while endothelial cells are very deeply stained. Tschaschin (20) has made an extensive study of these vitally staining cells, and also Aschoff and Kiyono (21). Tschaschin shows that these cells correspond to the resting wandering cells of Maximow, and a further source, especially in inflammation, he believes to be the hematogenous lymphocytes. These vitally staining cells he terms polyblasts. Anitschkow (19) believes that in part at least the fat-containing cells in the aortic lesions of cholesterol-fed rabbits are of this origin. He applies to them the term cholesterin ester-phagocytes. Aschoff and Kiyono (21) introduced the term histiocytes for these vitally staining cells, and believe that they are ultimately derived from the endothelium and certain cells of the reticulum of the blood-forming organs.

The fat-containing cells, especially in the early lesions, vary considerably in size, form, and fat content, and it seems possible to follow gradations from the spindle cells with a few fat droplets to the large cells filled with fat. As previously stated, it is believed that

the fibroblasts here as elsewhere play a part in taking up the fat, but from the appearance of gradations mentioned it does not necessarily follow that fibroblasts swell up to form the large cells described.

By this collection of cells an enormous thickening of the intima is produced, frequently to nearly twice the thickness of the media. In the earliest lesions observed the greater part of the fat was intracellular. A collection of fine fat droplets, apparently extracellular, is, however, sometimes seen lying along the inner surface of the internal layer of elastic tissue as described by Anitschkow. As the lesions progress there is a pronounced proliferation of fibroblasts and an abundant formation of collagen and fine elastic fibers, especially in the upper portion of the lesion. The large collection of fat-containing cells in the lower portion breaks down, and areas of degeneration are formed in the lower intima with an abundant deposit of cholesterol crystals, and in some cases calcification, capped by fibrous tissue (Fig. 2). Calcification was observed in the aortic lesions in four rabbits, Nos. 9, 11, 12, and 16. Two rabbits, 11 and 14, received calcium lactate with some of their feedings; Rabbit 11 about 0.5 gm. in each of 7 feedings, and Rabbit 14 a similar amount in 31 feedings. Since No. 11 showed a deposit of calcium, while No. 14 did not, and since three rabbits which received no additional calcium in their food showed such a deposit, it is evident that the addition of calcium to the rabbits' regular diet is not necessary to obtain calcification, and it would seem that the process is not facilitated by such addition.

Although the lesion is mainly intimal, it is not so limited. Even in the earlier cases some fat may be seen in the media, mostly in the upper third, but occasionally as deep as the middle, both in cells and extracellular between the layers of elastic tissue. In the more marked lesions the upper layers of elastic tissue are raised by the collection of fat and fat-containing cells in this region, and the same degeneration as described in the intima is seen here.

In Rabbits 12 and 16, which were fed longer than any of the other rabbits but with smaller doses, the aortic lesions differed somewhat from those in the other rabbits. While the intimal lesions were of the same character with many large fat-filled cells, the intimal thickening was not nearly as pronounced, fibrosis was slight, and areas of

degeneration with the deposit of cholesterol were not found. The media, however, was more markedly affected than in the rabbits receiving larger doses for a shorter period. Underlying the intimal lesions the media showed many nodular areas and longitudinal streaks, between the elastic layers, of granular fatty material with a considerable deposit of calcium salts. These were present in places, in Rabbit 12, as far as the middle of the media.

Wesselkin (9) noted lesions at the beginning of the pulmonary artery similar to those in the aorta. These lesions were present in eleven of fifteen rabbits in this series. In eight of these rabbits lesions were present not only at the beginning of the vessel but in its branches in the lungs. These lesions consisted of pronounced nodular intimal thickenings containing many large cells filled with fat (Fig. 3). They were very similar to those in the aorta. Degeneration with the deposit of cholesterol crystals, fibrosis, and calcification were present in some of the more pronounced lesions in the larger branches (Figs. 4 and 5), but were not as marked as in the aorta. The media was affected to a varying extent. In the severe cases some of the small branches showed similar intimal thickenings, occasionally so pronounced as apparently completely to obliterate the lumen.

The splenic lesions have been described by Ignatovski (22, 23) Anitschkow (24, 19), and Wesselkin (9). The findings in our series confirm in the main the observations of these authors. The spleen in Rabbit 5 was about three times normal size, in No. 15 somewhat over twice normal size. In five others there was moderate enlargement. Doubly refracting fat was present in all spleens except those of Nos. 2 and 3, but varied considerably in amount. The fat was contained in large cells in the pulp, similar large cells free in the venous sinuses, and in the endothelial lining cells of the sinuses. The cells free in the venous sinuses also contained considerable yellow granular pigment. The Malpighian bodies showed very little fat but an occasional large fat-containing cell was seen. Fat was sometimes present in small amount in the endothelium of the arteries, and their walls sometimes gave a diffuse orange stain with Sudan III. No intimal thickenings were observed. In the severe cases the fibroblasts in the capsule and trabeculæ showed a few fat droplets at either end of the nucleus.

Enlargement of the adrenals has been noted by several of the previously mentioned investigators and also by Rothschild (25), who reports experiments on the relationship of the adrenals to cholesterol metabolism and hypercholesterinemia. Enlargement of the adrenals appears to be a consistent finding, having been present in all rabbits except Nos. 2 and 3. In Rabbits 5 and 14 these organs were about four times the normal size. They were of a uniform, almost white, color except for a very small pinkish medullary portion. Beyond the excessive anisotropic fat content no lesions were observed.

The liver lesions have been especially studied by Chalatow (26). He obtained somewhat different results in rabbits fed on pure cholesterol from those in rabbits

fed on egg yolk. In the former the doubly refracting fat was mainly in the peripheries of the lobules and there was marked increase of connective tissue here, which, however, did not invade the lobule. In one case the fat was present in both the peripheries and centers of the lobules and there was an increase of connective tissue in both places. In the rabbits fed on egg the doubly refracting fat was in the centers of the lobules. In these rabbits there was an increase of periportal connective tissue, which, however, was more diffuse and invaded the lobules. He describes a marked proliferation of the bile ducts with subsequent regeneration of the liver cells from them. In the older cases the liver surface was irregular, but the organ was not reduced in size.

In our series the livers, with a few exceptions noted in the summary, were of a deep yellow color; they were of normal size or slightly enlarged, and the surface was always smooth. Doubly refracting fat was present in all cases except Nos. 2, 3, and 4; in most in very large amount. The author cannot confirm Chalatow's observation as to the different localization of the fat in the rabbits fed on cholesterol and those on egg yolk. No differences were noted between the two series either as to location of fat or production of connective tissue. A very large portion of the fat was situated in the central portions of the lobules. It was contained both in the Kupffer cells and in the parenchymal cells. Some fat was also present in the periportal spaces, contained in fibroblasts and large endothelial cells. There was a considerable degeneration of the liver cells about the central veins, even in the earlier cases. Small focal areas of degeneration were frequent in the neighborhood of the central veins or midway in the lobules. Cirrhosis was present in six rabbits, Nos. 5, 6, 7, 10, 11, and 12. In Nos. 5 and 12 there was a pronounced formation of fibrous tissue; in the other four the process was slight. In all these cases there was a formation of fibrous tissue about the portal spaces and also in the centers of the lobules. In the former region the connective tissue encroached on the periphery of the lobule, and small islands of liver cells were isolated by the fibrous overgrowth. Fibrous tissue was formed about the central veins and extended radially outward in fine strands into the lobule (Fig. 6). The small areas of focal degeneration mentioned become fibrous in the more pronounced cases.

Chalatow (26) believes that the proliferation of interlobular connective tissue occurs only in part as a reactive phenomenon, as is the case in atrophic liver

cirrhosis where proliferating connective tissue takes the place of liver cells in the peripheries of the lobules. In great part it proliferates, stimulated by the new-formed epithelium of bile ducts, to form a stroma for them.

It is true that the degenerated cells in the centers of the lobules and in the areas of focal degeneration are ultimately replaced by fibrous tissue. In the peripheries of the lobules, however, little if any degeneration of the liver cells is apparent, as a primary process at least, and in the author's rabbits the strands of cells in the connective tissue have the appearance of included liver cells rather than proliferating bile ducts. It is also worthy of note in this connection that the fat is present in the periportal areas in endothelial cells and fibroblasts. It would seem, therefore, that the fibrosis occurs as a result of the irritative action of the anisotropic fat on the connective tissue itself and its consequent proliferation rather than as a secondary reaction to the destruction of liver cells or for the purpose of forming a stroma for proliferating bile ducts, as believed by Chalatow.

Spontaneous cirrhosis is a common lesion in rabbits as pointed out by Ophüls (27), and it is possible that the periportal increase of connective tissue is in part to be thus accounted for. It is believed, however, that the intralobular formation of connective tissue, as seen in these rabbits, is not observed as a spontaneous lesion and there seems no room for doubt that this at least is due to the cholesterol feeding.

Little mention is made of the kidneys by any of the writers on the subject. Chalatow (28) says: "As the connective tissue phagocytes are scattered through all organs one can find in a rich circulation of cholesterol in the blood of rabbits, an isolated cell in nearly all organs which contains anisotropic fat. . . . . So one can, for example in the kidney in the interstitial cells, oftenest in the region of the collecting tubules, sometimes observe single cell elements of the type of connective tissue cells which are infiltrated with anisotropic fat." This author considers the process of anisotropic fatty infiltration of organs under two divisions: an infiltration of the stroma, in which the fat is present in phagocytic cells, which he terms xanthomatosis, and an infiltration of the parenchymatous cells, which he terms myelinosis. For the latter process he considers a preexisting deposit of isotropic fat to be necessary to bind the cholesterol, and he says (28): "Under the usual conditions a myelinosis of the kidney does not occur experimentally. And it is apparent why this is so, since in order to produce a myelinosis of the kidney it is necessary first to produce a deposit of isotropic fat in the kidney epithelium of longer or shorter duration. . . . . This, however, is very difficult to produce experimentally." Weltmann and Biach (12) had previously, with this same idea in mind, fed rabbits on cholesterol and in the course of the feeding injected uranium nitrate intraperitoneally. They thus obtained a deposit of anisotropic fat in the tubular epithelium.

The gross appearance of the kidneys in our series has been briefly given in the outline of the experiments. The kidneys of eight rabbits showed characteristic macroscopic lesions which, however, varied

considerably in severity. These consisted of opaque yellowish white stripes and nodules up to 1 mm. in breadth situated in the outer zone of the medulla, standing out sharply from the pink of the surrounding kidney tissue. In the more pronounced cases, Rabbits 5, 9, and 11, these were large and so numerous as to cover a large portion of the outer zone (Fig. 7). In these more severe cases fine yellow lines extended from the large lesions in the outer zone into the deeper portions of the medulla. In Rabbits 6, 7, 8, 10, and 13, lesions of the same character were present, but smaller and fewer in number.

These yellow lesions are due to a localized deposit of anisotropic fat. The kidneys show a small amount of doubly refracting fat in the tubular epithelium, which, however, is not confined to any particular region, but is found in the tubules of both cortex and medulla. Whether or not a neutral fatty degeneration preexisted would be difficult to decide. The striking lesion microscopically in these kidneys, however, and that to which the nodular lesions seen in the gross are due is not that of the tubules but of the interstitial tissue. Accepting the classification of Chalatow, it is a xanthomatosis rather than a myelinosis. The fat is contained for the most part in endothelial cells, both those of the capillaries and those of the lymph spaces, but especially the latter. The fat is seen in the endothelial cells of the capillaries to some extent throughout the kidneys, less abundant, however, in the capillaries of the glomeruli and elsewhere in the cortex than in the medulla. The fat so situated is small in amount, a few fine drops being seen in the cells at either end of the nucleus. Three rabbits, Nos. 12, 14, and 16, in addition to the eight mentioned above, showed this diffuse distribution of fat but without the nodular lesions seen in the latter. These nodular deposits are confined to the outer zone of the medulla in the less pronounced cases; in the more severe they extend into the deeper medulla but never outward into the cortex. In these areas the endothelial cells of the lymph spaces appear as large round or irregular cells filled with doubly refracting fat, very similar in appearance to the cells described in the intima of the aorta (Fig. 8). In more pronounced cases there are large collections of these swollen anisotropic fat-filled cells. In two rabbits, 9 and 11, many of these lesions show in their centers large areas of degeneration of the fat-containing cells, with an abun-

dant deposit of cholesterol crystals (Fig. 9). In No. 11 there is also some deposit of calcium in these degenerated areas. The tubules are for the most part destroyed, but an occasional collapsed tubule is seen passing through the lesions. Some of the capillaries in the deeper medulla, underlying these lesions, contain large numbers of round swollen cells filled with fat (Fig. 10). In only one rabbit, No. 11, a single nodular lesion of the intima of one of the larger vessels in the cortex was found, similar to those described in the vessels of the lung. The kidney lesions, therefore, do not appear to be due to lesions of the large vessels.

That some relationship exists between scars in these kidneys and the cholesterol lesions is evident. It will be noted from the outline that of the eight rabbits whose kidneys showed cholesterol lesions in the gross, six showed also scars on the surface of the kidneys, and that none of the kidneys of the other rabbits showed scars. These scars are similar in appearance to those seen in the so called spontaneous nephritis of rabbits, appearing in the gross as small, slightly depressed, purplish areas, and microscopically as cellular or fibrous interstitial lesions with collapse or disappearance of the tubules. In these kidneys nearly every scar shows at its base, in the outer zone of the medulla, a cholesterol lesion as described above. These collections of large fat-containing cells are never seen in the scars in the cortex but always at their base in the medulla. Rarely a scar is seen in the cortex at whose base no cholesterol lesion is apparent in the sections as cut; and occasionally a cholesterol lesion is seen with no overlying scar in the cortex. In Rabbit 8 scars extending through the cortex are not seen either in the gross or microscopically. There are, however, a considerable number of cholesterol lesions in the outer zone of the medulla. These are early; that is, they are made up of large fat-containing cells without areas of degeneration. Between the large cells are seen a few connective tissue fibrils. In Rabbit 7 there are no scars in the cortex. In the outer zone of the medulla, situated between the tubules, are a few very small longitudinal cellular scars. These show none of the large fat-containing cells described, but many spindle cells, apparently fibroblasts, contain a few fat droplets.

From the appearance of the tissue immediately surrounding the

older lesions it seems apparent that the cholesterol deposited in these areas in the kidney produces some proliferation of fibroblasts as it does in the aorta and in the liver. Whether, however, the cholesterol lesions are responsible for the scars in the cortex or whether the scars preexisted, is an important but difficult matter to decide from these few rabbits. If the scars preexisted the percentage of spontaneous nephritis in these rabbits was very high. On the other hand, it is to be noticed that a few scars were seen at whose base no cholesterol lesions were found, that in one case, No. 13, the scarring was very pronounced in a rabbit which had been fed for but 34 days, and that two rabbits, Nos. 12 and 15, whose kidneys showed no scars, and who, from a comparison with other rabbits, would seem to have been fed a sufficient amount of cholesterol and for a sufficient length of time to produce lesions, nevertheless showed none. These facts are in favor of the view that the scars preexisted and that a preexistent scar or perhaps merely a nodular infiltration of endothelial cells is necessary for the formation of these lesions. If this is so it remains to be explained why the large anisotropic fat-containing cells do not appear and cholesterol lesions are not formed in scars or portions of scars lying in the cortex. Small scars in the cortex about the glomeruli and elsewhere show no lesions, and scars extending through the cortex show cholesterol lesions only at their base in the medulla.

Considering the results as a whole it is found that the feeding of cholesterol-containing food to rabbits produces a widely distributed deposit of doubly refracting fat. This process is primarily one of infiltration, not of degeneration. For the production of such a deposit exceedingly large doses of cholesterol are necessary relative to the rabbit's body weight. This is shown by the results in Rabbits 2, 3, and 4, which were purposely given small doses to test this point. In none of these rabbits were any lesions found or any deposit of doubly refracting fat, except in the adrenals, which in No. 4 were slightly enlarged, and in the spleen of No. 4 which showed a very few large cells in the pulp containing the fat. Where this fat is deposited in large amounts, characteristic lesions are secondarily produced, notably an atheroma of the aorta, lesions of other vessels, mainly intimal, especially of the branches of the pulmonary artery, a cirrhosis of the liver, and lesions in the kidney as described.

Aschoff (16), Anitschkow (24, 17), Zinserling (29), and others discuss the possible relationship between the aortic lesion and human atheroma. Such a discussion would necessarily be at the present time largely theoretical and beyond the province of this paper. It seems, however, worthy of note and of possible importance for human pathology that in the rabbit an experimentally produced deposit of doubly refracting fat in the aorta finally results in a lesion practically identical with that seen in human atheroma. On the other hand, these lesions in the aorta are constantly associated with conditions in the spleen, liver, and pulmonary vessels which are not commonly found in human atheroma, but which seem to be an important part of the general picture of an overloading of the animal body with cholesterol. These additional manifestations could not be eliminated by feeding smaller doses. The rabbit is evidently an animal unable to cope with large doses of cholesterol, although small doses are comparatively innocuous.

SUMMARY.

The feeding of egg yolk or pure cholesterol to rabbits produces an abundant deposit of anisotropic fat in various organs. From this deposit characteristic lesions secondarily result in certain organs. Prominent among these lesions is an atheroma of the aorta very similar in the gross and histologically to the human lesion. Lesions of other vessels are also produced, conspicuous among which are those of the branches of the pulmonary artery. There is a large deposit of anisotropic fat in the liver which produces a cirrhosis. Enlargement of the adrenals occurs, probably due to the storage of an excessive amount of anisotropic fat. In a certain proportion of rabbits conspicuous lesions are produced in the kidneys consisting of nodular deposits of anisotropic fat in the medullary portion, the fat being contained for the most part in endothelial cells and fibroblasts in the interstitial tissue. Later softening occurs in these areas as in the aorta; the cells break down; there is an abundant deposit of cholesterol crystals, some calcification, and a proliferation of connective tissue. Scars frequently extend outward from these lesions through the cortex, but the nodular deposits of anisotropic fat are

never seen in the cortex. It is impossible to determine definitely from these experiments whether or not these deposits are dependent on preexisting interstitial lesions.

In conclusion I wish to acknowledge my indebtedness to Dr. Ophüls for his advice and assistance in this work, and to Dr. Jean Oliver for suggestions and for his assistance in taking the photomicrographs.

BIBLIOGRAPHY.

1. Ignatovski, A. I., *Izviest Imp. Voyenno-Med. Akad., S. Peterb.*, 1908, xvi, 154.
2. Starokadomsky, L. M., and Ssobolew, L. W., *Frankfurt. Ztschr. f. Path.*, 1909, iii, 912.
3. d'Amato, L., *Virchows Arch. f. path. Anat.*, 1908, cxcii, 86.
4. Garnier, M., and Simon, L.-G., *Compt. rend. Soc. de biol.*, 1907, lxiii, 250.
5. Stuckey, N. W., *Centralbl. f. allg. Path. u. path. Anat.*, 1910, xxi, 668.
6. Stuckey, N. W., *Centralbl. f. allg. Path. u. path. Anat.*, 1911, xxii, 379.
7. Stuckey, N. W., *Centralbl. f. allg. Path. u. path. Anat.*, 1912, xxiii, 910.
8. Chalatow, S. S., *Virchows Arch. f. path. Anat.*, 1912, ccvii, 452.
9. Wesselkin, N. W., *Virchows Arch. f. path. Anat.*, 1913, ccxii, 225.
10. Anitschkow, N., and Chalatow, S., *Centralbl. f. allg. Path. u. path. Anat.*, 1913, xxiv, 1.
11. Wacker, L., and Hueck, W., *München. med. Wchnschr.*, 1913, lx, 2097.
12. Weltmann, O., and Biach, P., *Ztschr. f. exper. Path. u. Therap.*, 1913, xiv, 367.
13. van Leersum, E. C., *Virchows Arch. f. path. Anat.*, 1914, ccxvii, 452.
14. Anitschkow, N., *Beitr. z. path. Anat. u. z. allg. Path.*, 1913, lvi, 379.
15. Adler, I., *Jour. Exper. Med.*, 1914, xx, 93.
16. Aschoff, L., *Beihefte z. med. Klin.*, 1914, x, No. 1.
17. Anitschkow, N., *Beitr. z. path. Anat. u. z. allg. Path.*, 1914, lix, 306.
18. Klotz, O., *Centralbl. f. allg. Path. u. path. Anat.*, 1908, xix, 535.
19. Anitschkow, *Beitr. z. path. Anat. u. z. allg. Path.*, 1914, lvii, 201.
20. Tschaschin, S., *Folia Haematol.*, 1913–14, xvii, 317.
21. Kiyono, K., Die vitale Karminspeicherung, Jena, 1914.
22. Ignatovski, *Izviest Imp. Voyenno-Med. Akad., S. Peterb.*, 1908, xviii, 231.
23. Ignatovski, A., *Virchows Arch. f. path. Anat.*, 1909, cxcviii, 248.
24. Anitschkow, *Deutsch. med. Wchnschr.*, 1913, xxxix, 741.
25. Rothschild, M. A., *Beitr. z. path. Anat. u. z. allg. Path.*, 1914, lx, 39.
26. Chalatow, *Beitr. z. path. Anat. u. z. allg. Path.*, 1914, lvii, 85.
27. Ophüls, W., *Proc. Soc. Exper. Biol. and Med.*, 1909–11, vii, 75.
28. Chalatow, *Virchows Arch. f. path. Anat.*, 1914, ccxvii, 351.
29. Zinserling, W. D., *Centralbl. f. allg. Path. u. path. Anat.*, 1913, xxiv, 627.

EXPLANATION OF PLATES.

Plate 15.

Fig. 1. Rabbit 9. Thoracic portion of aorta. Shows large elevated plaques in the upper part; smaller lesions below.

Fig. 2. Rabbit 9. Aorta. Shows the edge of an area of atheroma with fibrosis on the surface and degeneration with cholesterol crystal spaces below. Slight invasion of media.

Plate 16.

Fig. 3. Rabbit 11. Branch of pulmonary artery. Shows a pronounced nodular intimal thickening made up of the large cells which contain the anisotropic fat.

Fig. 4. Rabbit 9. Branch of pulmonary artery. Shows the large cells and calcification in an intimal lesion.

Plate 17.

Fig. 5. Rabbit 9. Branch of pulmonary artery. Shows lesions composed of large cells, with fibrosis on surface, cholesterol crystal spaces, and some calcification below. Mainly intimal, but invading media.

Fig. 6. Rabbit 5. Liver. Shows a central vein with fine connective tissue fibrils extending outward into the lobule.

Plate 18.

Fig. 7. Rabbit 9. Kidney. Shows the scars on the surface and the nodular lesions involving a large portion of the outer zone of the medulla and extending as fine lines into the inner zone.

Fig. 8. Rabbit 5. Kidney. Shows one of the nodular lesions in the outer zone of the medulla, which consist of large cells loaded with anisotropic fat.

Plate 19.

Fig. 9. Rabbit 11. Kidney. One of the more pronounced lesions in the outer zone of the medulla. Many of the large cells are seen which contain the anisotropic fat; also areas of degeneration with numerous cholesterol crystal spaces. Surrounding the lesion there is moderate fibrosis. In the lesion a few collapsed tubules still persist.

Fig. 10. Rabbit 5. Kidney. Shows a capillary in the inner zone of the medulla containing many large cells. These cells are filled with anisotropic fat.

THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

XIV. Changes in the Blood Following Diversion of the Splenic Blood from the Liver. A Control Study of the Effects of Splenectomy.

By EDWARD B. KRUMBHAAR, M.D., JOHN H. MUSSER, Jr., M.D., and MAX M. PEET, M.D.

(*From the John Herr Musser Department of Research Medicine of the University of Pennsylvania, Philadelphia.*)

(Received for publication, July 17, 1915.)

In the earlier publications from this laboratory (1) have been described the various phenomena which follow splenectomy in the dog. In the discussion of the most striking of these,—anemia, increased resistance of the red cells, decreased tendency to jaundice, and prolongation of the recovery from anemia—it was assumed that, inasmuch as the spleen was absent, these changes were probably due to the loss of some function peculiar to this organ. As to the exact nature of the function lost, no definite opinion has thus far been given. Three possibilities, however, readily suggest themselves: (1) the loss of some function of blood destruction or regeneration resident in the spleen itself; (2) the loss of an internal secretion acting on the distant hemopoietic tissues, such as the bone marrow; and (3) the alteration of the venous drainage of the spleen, which, in that it is an important source of the portal blood, may have an essential relation to some peculiar function of the liver.

It is obvious that the value of some of these hypotheses might be tested without removal of the spleen, by diverting its venous outflow to the liver. We have, therefore, repeated many of our experiments, but instead of removing the spleen, the splenic vein has either been ligated or transplanted into the vena cava, or an Eck fistula has been established. As far as we know, exactly similar experiments

have not previously been attempted, though two observations have a bearing on the problem. Contrary to our results, Nassau (2) found that the number, color, and resistance of the red cells remained unchanged after simple Eck fistula; but as he gives only one count before and one after the operation, his observations are not conclusive. The other report which concerns us is Pribram's (3) artificial constriction of the splenic vein with resultant passive congestion of the spleen. The slight anemia that this caused is analogous to some of the results we report below.

If the anemia following splenectomy is caused by the removal with the spleen of some necessary factor in blood formation or of a hormone essential to the hemopoietic tissues, this factor should not operate in the vein transplantation experiments and Eck fistula, unless it is also essential that such a substance have direct approach to the liver for its proper functioning or activation. If, on the other hand, the anemia is largely due to the interference with the supply of splenic blood to the liver, it should occur in all animals tested. These problems, and also that concerning the influence of the experimental procedures on increased resistance of the red cells, will be considered in this paper. Other problems such as the decreased tendency to jaundice and the prolonged repair of anemia in the splenectomized animal when hemolytic agents are given, will be presented in a later communication.

Methods.

In the ligation experiments it was found necessary, on account of the numerous branches and the anastomoses of the splenic vein with veins from the stomach, to tie all branches of the splenic vein shortly after they left the spleen. In the earlier operations one of the subdivisions of the artery was also ligated, to lessen the supposed danger of rupture. This, later, was abandoned when it was found to be unnecessary, as well as undesirable, on account of infarct production. Postmortem examination of animals subjected to this operation showed in each case that all branches had been tied, that the vessel beyond the ligation was much diminished in size or completely obliterated, and that there was usually little attempt at compensatory development of new veins from the adherent omentum. When such

new-formed veins were present it was found that they seldom emptied into the portal system, and therefore, as a rule, did not complicate the experiment. Eck fistula was performed in the usual manner, the proximal end of the portal vein being tied off above its new anastomosis with the vena cava just before its entrance into the liver. Transplantation of the splenic vein into the inferior vena cava presented considerable difficulty on account of the smallness of the vein and the necessity of stretching it a little to make it reach the vena cava. In only one experiment, however, was it found that the transplanted vein had been occluded by thrombus. The success of the operation was always determined by examination of the vessels at autopsy, and in some instances injection specimens were prepared and dissected to make doubly sure that no new anastomosis had formed.

The particular phenomena studied were: (1) the quantitative changes in the red blood cells, leukocytes, and hemoglobin; (2) the resistance of the red cells to hypotonic salt solution; (3) the general condition as indicated by the weight of the animals and condition of the urine; and (4) the gross and microscopical appearance of the spleen and other viscera at autopsy.

The dogs have been kept on the usual mixed diet of table scraps. This has been shown to maintain properly normal dogs as well as those convalescent from operations other than splenectomy, without the development of anemia (4).

RESULTS.

Our studies have been made on 15 dogs, divided as follows: ligation of splenic vein, 4; transplantation of splenic vein, 4; Eck fistula, 3; and, as controls, splenectomy, 3; transplantation of the inferior mesenteric vein, 1. In the case of the last dog, it was intended to transplant the splenic vein, and it was not until autopsy that we found that the inferior mesenteric vein had been used by mistake. As some of these dogs were used also for the study of the effect of hemolytic agents, all are not presented in this paper.

Changes in the Spleen.

In those dogs in which all branches of the splenic vein had been ligated and in which adequate new venous channels have failed to develop, the spleen shows considerable change. Before the operation is completed the spleen increases to almost double its size and takes on a dark purple color. If the animal is allowed to survive two or more months, the spleen at autopsy is found to be considerably smaller and much firmer than normal. The capsule is slightly thickened and puckered and the organ has a pale bluish color. On section the tissue cuts with increased resistance and a few small shrunken infarcts are occasionally found. The cut surface is less bloody than usual and shows an increase of fibrous tissue, with indistinct Malpighian corpuscles. Histologically, the tissue appears to be condensed with collapsed sinuses rather than to exhibit any marked fibrous tissue hyperplasia. Some hemosiderin pigment is present, the Malpighian corpuscles are small, and here and there are several areas of hyaline degeneration, which do not respond to the test for amyloid. Thromboses are not found, except in connection with the old postoperative infarcts. In the earlier animals, in which a branch of the artery was also ligated, a bulging hemorrhagic infarct appeared in the corresponding area. The abdominal lymph nodes and the liver are apparently unchanged. Speculation as to the adjustment of the splenic circulation in these cases must remain unsatisfied. The most probable explanation seems to be that the elastic spleen is able not only to accommodate the increasing pressure without rupture, but, with the aid of the minute new-formed capillaries in the adherent omentum, to maintain sufficient degree of nutrition to prevent necrosis. We have seen, however, that the exchange is not sufficient to prevent atrophy.

At the time of the operations for Eck fistula and the splenic vein-vena cava anastomosis, there is necessarily a temporary occlusion of the large veins. The spleen and intestine become very dark and turgid, but when the clamps are removed, these organs quickly return to normal color. At autopsy on animals so treated, except for numerous adhesions and frequently perisplenitis, no noteworthy changes are found.

Changes in the Blood.

An example of the changes in the blood which occur after ligation of the splenic vein is given in Table I.

It may be seen that this animal developed a moderate degree of anemia lasting several weeks and similar to but less severe than that following splenectomy. The anemia is accompanied by a temporary slight increase in resistance of the red cells to hypotonic salt solution.

TABLE I.

Blood Changes Following Ligation of Splenic Veins.

Dog 47.	Hemoglobin.	Red blood cells per cmm.	Hemolysis.		Leukocytes per cmm.	Polymorphonuclears.	Small lymphocytes.	Large transitionals.	Eosinophils.	Weight.
			Begins.	Complete.						
	per cent									*kilos*
Before	102	6,275,000	0.475	0.35	9,800	7,200	700	1,800	100	6.0
1 day after	98	6,110,000			19,000	15,200	950	2,850	0	5.7
4 days "	92	5,350,000	0.475	0.275	16,500					5.6
1 wk. "	90	5,520,000	0.425	0.275	16,800	13,600	1,300	1,600	300	5.4
2 " "	72	4,900,000	0.425	0.3	18,800	15,400	1,900	1,500	0	
3 " "	70	4,600,000	0.45	0.3	21,000	16,800	3,100	300	300	
4 " "	78	5,000,000			18,600					6.4
5 " "	74	4,380,000			15,000					
7 " "	80	5,040,000	0.475	0.275	12,800	10,000	1,700	1,100	0	7.3
10 " "	82	5,050,000	0.45	0.3	12,400					
12 " "	95	5,830,000	0.475	0.325	14,800	9,500	3,400	1,500	400	7.5
13 " "	92	6,410,000	0.475	0.35	14,900	9,200	3,600	1,200	900	
14 " "	95	6,150,000			15,600	12,200	2,500	600	100	7.4

As in splenectomy, there is an immediate leukocytosis, due to polymorphonuclear and transitional forms, but prolonged by a more persistent lymphocytosis and eosinophilia. A very slight drop in weight occurs after operation, but later there is a distinct increase above the original weight. It has been shown elsewhere (4) by the study of control operations such as nephrectomy, that the changes here described are not merely postoperative. Other dogs with ligated veins gave similar results; such as Dog 51, with a maximum drop of 14 per cent

of hemoglobin and 1,200,000 red cells per cmm.; Dog 74, hemoglobin loss 24 per cent, red blood cell loss 1,800,000 per cmm.

The effect of diverting the splenic venous blood from the liver by transplanting the splenic vein into the vena cava is shown in Table II.

Much the same changes as after ligation are found here. In another dog (No. 71) similarly treated, the increased resistance was much more marked: before operation, hemolysis began at 0.50 and was complete at 0.35; soon after operation, it began at 0.425 and was complete at 0.25. Anemia was also present (a drop of 20 per cent in hemoglobin and of 1,800,000 red blood cells). A third animal (No. 16) lost 35 per cent hemoglobin and over 2,000,000 erythrocytes, and a fourth (No. 24) 22 per cent hemoglobin and over 1,000,000 erythrocytes.

The changes in an Eck fistula experiment are seen in Table III.

In another Eck fistula dog (No. 68), the increased resistance of the red cells was more marked (before operation, hemolysis began at 0.45 and was complete at 0.3; soon after operation it began at 0.425 and was complete at 0.225) and the animal lost 33 per cent hemoglobin and 2,000,000 red blood cells, in four weeks' time after operation. In a third (No. 5) with an even greater anemia, the blood picture was complicated by the occurrence of infection, so the figures are not given.

That interference with inflow to the portal circulation, from organs other than the spleen is responsible for changes in the blood is shown in another experiment (Table IV) in which by accident an anastomosis of the inferior mesenteric vein was done.

This dog developed an anemia of mild grade and recovered from it sooner than did the other dogs of this series. Although little weight can be placed on a single experiment such as this, the observation tends to support the theory that the supply of portal blood to the liver is an important factor in our problem.

The urine remained without bile in all the animals used.

There was an occasional postoperative loss of weight (especially in some of the Eck fistula dogs), but this was by no means constant, and bore no apparent relation to the anemia. This was frequently followed by an increase to more than the original weight, just as is frequently found after splenectomy.

TABLE II.

Blood Changes Following Transplantation of the Splenic Vein.

Dog 4.	Hemoglobin.	Red blood cells per cmm.	Hemolysis.		Leukocytes per cmm.	Polymorpho-nuclears.	Small lympho-cytes.	Large transi-tionals.	Eosinophils.	Weight.
			Begins.	Com-plete.						
	per cent									*kilos*
Before	97	6,960,000	0.45	0.35	16,100	8,400	4,700	1,600	1,400	9.4
1 day after	104	7,040,000	0.425	0.3	34,000	29,600	3,100	1,300	0	
1 wk. "	75	5,550,000	0.45	0.3	19,300	12,400	3,700	1,900	1,300	9.7
2 " "	68	5,020,000	0.475	0.275	16,900	11,800	2,700	2,200	200	9.4
3 " "	70	4,530,000								9.6
4 " "	80	4,510,000	0.475	0.3	14,700	9,600	2,800	1,500	800	10.4
5 " "	74	5,440,000	0.475	0.325	14,000	9,800	2,800	420	1,080	10.5
6 " "	78	5,630,000	0.45	0.3	11,200	6,300	4,000	560	360	
7 " "	82	5,770,000	0.45	0.325	11,100	7,400	2,900	200	600	12.9
8 " "	85	6,060,000	0.45	0.325						
9 " "	96	6,500,000								13.2

TABLE III.

Blood Changes Following Eck Fistula.

Dog 31.	Hemoglobin.	Red blood cells per cmm.	Hemolysis.		Leukocytes per cmm.	Polymorpho-nuclears.	Small lympho-cytes.	Large transi-tionals.	Eosinophils.	Weight.
			Begins.	Com-plete.						
	per cent									*kilos*
Before	99	6,500,000	0.45	0.325	13,200	9,800	2,700	500	200	16.1
1 day after	88	6,040,000	0.475	0.325	36,000	32,400	2,800	800	0	
3 days "	84	6,300,000	0.45	0.325	32,400	27,500	2,900	1,600	400	14.2
5 " "	72	5,400,000	0.425	0.3	24,000	17,300	3,800	2,400	500	14.2
1 wk. "	74	5,500,000	0.425	0.3	18,800	13,700	3,400	800	900	13.6
2 " "	68	5,040,000	0.425	0.275	19,600	15,500	3,900	600	600	13.5
3 " "	73	5,110,000	0.425	0.275						
4 " "	67	4,460,000	0.425	0.3	21,000	15,500	4,400	600	500	14.6
5 " "	72	4,880,000	0.475	0.325	18,400	12,900	3,700	600	1,200	14.2
6 " "	84	5,600,000	0.475	0.325	16,000	10,500	3,800	400	1,300	14.9
7 " "	90	6,200,000	0.45	0.3	15,200	10,300	2,800	600	1,500	15.3
8 " "	98	6,520,000	0.45	0.3	15,800	11,400	3,500	300	600	15.4

TABLE IV.

Blood Changes Following Transplantation of Inferior Mesenteric Vein.

Dog 55.	Hemo-globin.	Red blood cells per cmm.	Hemolysis.		Weight.
			Begins.	Complete.	
	per cent				*kilos*
Before	100	5,940,000	0.45	0.3	14.7
2 days after	76	5,240,000	0.4	0.25	
3 " "	75	5,100,000	0.4	0.25	13.9
10 " "	76	4,880,000	0.45	0.275	
2 wks. "	85	5,400,000	0.425	0.25	
3 " "	84	5,890,000	0.425	0.25	
4 " "	78	5,600,000			14.7
5 " "	88	5,720,000			
6 " "	92	5,800,000	0.425	0.275	15.3

SUMMARY.

1. In dogs whose splenic veins have been ligated or transplanted into the inferior vena cava, or in which an Eck fistula has been made, an anemia occurs which resembles that following splenectomy and shows the same general variation in degree and duration.

2. The resistance of the red cells to hypotonic salt solution is quickly increased, sometimes coincident with and sometimes preceding the anemia. As a rule, it gradually returns to normal in about the same length of time as it takes the anemia to disappear, but may remain increased for longer periods.

3. There is an initial leukocytosis, involving at first the polymorphonuclear leucocytes and transitional cells. As the total leukocytosis diminishes there is both a relative and actual increase of small lymphocytes and usually of eosinophils. This may either be temporary or last during the rest of the period of observation and differs from the ordinary postoperative leukocytosis.

4. Ligation of the splenic vein is followed by considerable atrophy of the spleen, but not by necrosis or thrombosis. There is rarely adequate new vein formation. The other operations cause little or no change in the spleen.

5. Whether the disturbances as described are due to the loss of a certain volume of blood to the liver, or, as has been previously

suggested, to the loss of a splenic hormone, it is impossible to say. If the former is true, the method of production of the anemia still remains unexplained. It is evident, furthermore, that the latter theory has also no value unless it is assumed that this hormone must be activated by passage through the liver.

BIBLIOGRAPHY.

1. Pearce, R. M., Austin, J. H., and Krumbhaar, E. B., The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. I. Reactions to Hemolytic Serum at Various Intervals after Splenectomy, *Jour. Exper. Med.*, 1912, xvi, 363. Pearce, R. M., Austin, J. H., and Eisenbrey, A. B., II. The Relation of Hemoglobinemia to Hemoglobinuria and Jaundice in Normal and Splenectomized Animals, *Jour. Exper. Med.*, 1912, xvi, 375. Pearce, R. M., Austin, J. H., and Musser, J. H., Jr., III. The Changes in the Blood Following Splenectomy and Their Relation to the Production of Hemolytic Jaundice, *Jour. Exper. Med.*, 1912, xvi, 758. Pearce, R. M., and Austin, J. H., V. Changes in the Endothelial Cells of the Lymph Nodes and Liver in Splenectomized Animals Receiving Hemolytic Serum, *Jour. Exper. Med.*, 1912, xvi, 780. Musser, J. H., Jr., and Krumbhaar, E. B., VI. The Blood Picture at Various Periods after Splenectomy, *Jour. Exper. Med.*, 1913, xviii, 487. Pearce, R. M., and Peet, M. M., VII. The Effect of Hemolytic Serum in Splenectomized Dogs, *Jour. Exper. Med.*, 1913, xviii, 494. Krumbhaar, E. B., Musser, J. H., Jr., and Pearce, R. M., VIII. Regeneration of the Blood of Splenectomized Dogs after the Administration of Hemolytic Agents, *Jour. Exper. Med.*, 1913, xviii, 665. Pearce, R. M., and Pepper, O. H. P., IX. The Changes in the Bone Marrow after Splenectomy, *Jour. Exper. Med.*, 1914, xx, 19. Krumbhaar, E. B., and Musser, J. H., Jr., X. Concerning the Supposed Regulatory Influence of the Spleen in the Formation and Destruction of Erythrocytes, *Jour. Exper. Med.*, 1914, xx, 108. Austin, J. H., and Pearce, R. M., XI. The Influence of the Spleen on Iron Metabolism, *Jour. Exper. Med.*, 1914, xx, 122.
2. Nassau, E., Das Blutbild beim Hunde mit Eckscher Fistel, *Arch. f. exper. Path. u. Pharmakol.*, 1914, lxxv, 123.
3. Pribram, B., Hypersplenische Hämophthisen und Stauungsmilz, *Wien. klin. Wchnschr.*, 1913, xxvi, 1607.
4. Pearce, R. M., Austin, J. H., and Pepper, O. H. P., XV. The Influence of Diet upon the Anemia Following Splenectomy, *Jour. Exper. Med.*, 1916, xxii, 682.

THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

XV. The Resistance to Hemolytic Agents of Dogs in Which the Splenic Blood Has Been Diverted from the Liver.

By EDWARD B. KRUMBHAAR, M.D., and JOHN H. MUSSER, Jr., M.D.

(*From the John Herr Musser Department of Research Medicine of the University of Pennsylvania, Philadelphia.*)

(Received for publication, July 17, 1915.)

In a previous paper (1) has been discussed the effect upon the blood picture of three methods (transplantation of the splenic vein into the vena cava, Eck fistula, and ligation of the splenic veins) of diverting the splenic blood from the liver. In other papers (2) of this series have been described the decreased tendency to jaundice in splenectomized dogs receiving hemolytic serum and the slow repair of the accompanying anemia. In the present paper we shall present the results of a study of these two phenomena in animals whose splenic blood supply has been diverted from the liver, as above described.

Methods.

As hemolytic agents we have employed both toluylenediamin and hemolytic immune serum. The hemolytic serum was prepared by injecting at regular intervals the erythrocytes of the dog into rabbits. The serum so prepared was always injected intravenously into the dog under ether anesthesia. The other hemolytic agent, Merck's meta toluylenediamin, was given by stomach tube and in adequate doses never failed to cause anemia and jaundice. In each experiment the control animal received the same proportionate amount of drug or serum per kilo as did the test animals. Dogs of approximately the same size were selected and, on account of the occasional necessity of catheterization, female

dogs were used when possible. Splenectomized as well as normal animals were included in the series to further comparison with our previous results. Daily and later weekly examinations of urine and complete blood examinations (including resistance of erythrocytes to hypotonic salt solution) were made and records of weight were kept. Each animal was studied as to condition of urine and blood before the experiment began. Both cage urine and catheterized specimens (in doubtful cases) were examined, and the presence of bile pigment in the urine, as determined by Gmelin's and Rosenbach's tests, was taken as the surest evidence of jaundice. At the termination of the experiment the test animals were killed in order to determine the exact anatomical disturbance caused by the operation.

RESULTS.

Decreased Tendency to Jaundice.

In the animals with ligation of the splenic vein, it was occasionally found at autopsy that a small number of new blood vessels had forced their way through the splenic attachments, thus defeating the object of the experiment. In a previous paper we stated that new vein formation was inadequate to prevent atrophy of the spleen or the development of anemia. The mechanical vascular connection with the liver once being reestablished, however, these animals are apparently able to react with well marked jaundice as do normal dogs.

As may be seen in Table I, in all animals receiving adequate toluylenediamin, control as well as test dogs, some bile appeared in the urine. Frequently also well marked staining of the skin and scleræ persisted for many days. The bile was less in amount, however, and lasted for shorter periods in the test animals than in the normal controls. In fact, the animals with the vein transplant and Eck fistula showed even less tendency to jaundice than some of the splenectomized animals. Increased resistance of the red cells to hypotonic salt solution and the presence of anemia also contributed to diminish the jaundice; but it was shown that lessened tendency to jaundice might nevertheless be present in the absence of these features. For example, Dog 71, which had completely recovered from its anemia, showed the least tendency to jaundice, and Dog 79 which

TABLE I.

Jaundice after Administration of Toluylenediamin (0.34 Gm. per Kilo).

Time after administration.	Dog 71. Splenic vein transplant (2 mos.).	Dog 79. Splenectomy (1½ mos.).	Dog 51. Ligation (2 mos.).	Dog 75. Normal control.
	0 425 B. H.–0.325 C. H.* No anemia.	0.40 B.H.–0.275 C. H. No anemia.	0.45 B. H.–0.275 C. H. Slight anemia.	0.425 B. H.–0.275 C. H.
Before	Urine normal.	Urine normal.	Urine normal.	Urine normal.
1 day after	Bile trace.	Bile slight.	Bile moderate.	Bile marked.
3 days "	" heavy trace.	" "	" "	" "
5 " "	" trace.	" moderate.	" "	" "
7 " "	" absent.	" "	" "	" "
9 " "	" "	" "	" "	" moderate.
11 " "	" "	" trace.	" faint trace.	" trace.
13 " "	" "	" "	" absent.	" "
15 " "	" "	" "	" "	" "
17 " "	" "	" absent.	" "	" absent.

* These figures in this and other tables indicate strength of salt solution at which hemolysis occurs; B. H, indicates beginning hemolysis, C. H. complete hemolysis. Increasing amounts of bile in the urine are expressed in the following terms: faint trace, trace, heavy trace, slight, moderate, marked.

had the most resistant red cells, showed the same tendency to jaundice as did the control.

In another experiment (Table II), in which a smaller single dose was administered, similar results were obtained. It was in this

TABLE II.

Jaundice after Administration of Toluylenediamin (0.13 Gm. per Kilo).

Time after administration.	Dog 68. Eck fistula (1 mo.).	Dog 55. Mesenteric vein transplant (1½ mos.).	Dog 77. Normal control.
	0.4 B.H.–0.275 C. H. Anemia present.	0.425 B. H.–0.275 C. H. No anemia.	0.425 B. H.–0 35 C. H.
Before	Urine normal.	Urine normal.	Urine normal.
1 day after	Bile absent.	Bile trace.	Bile trace.
2 days "	" faint trace.	" "	" marked.
3 " "	" " "	" moderate.	" moderate.
5 " "	" absent.	" doubtful.	" "
7 " "	" "	" absent.	" trace.
9 " "	" "	" "	" absent.

experiment that an animal that was supposed to have had the splenic vein transplanted was found at autopsy to have a branch of the inferior mesenteric vein transplanted by mistake, thus unwittingly causing an excellent double control for the Eck fistula test (No. 68). The increased resistance to jaundice in this dog (No. 55) as compared to the normal (No. 77) is still further evidence of the importance of the mechanical factor of blood supply to the liver.

In a third experiment, in which three smaller repeated doses were given to animals with (1) splenic vein transplant; (2) splenic vein ligation; and (3) a normal control, these results were confirmed. The vein transplant dog, although his red cells at the time were the least resistant of the three, failed to get jaundice; in the other two it did occur. The jaundice in the ligation experiment, however, lasted three days and that of the control nine days,

In the experiments with hemolytic immune serum the same results were obtained. In a preliminary experiment, in which large doses of serum were given, an Eck fistula dog alone survived and developed jaundice; one with the vein transplant and the control died in a few hours; the control, however, had already developed hemoglobinuria, whereas the test dog failed to develop either hemoglobinuria or jaundice.

With a weaker serum (Table III) the greater resistance of the vein transplant animal is shown. A result contrary to our former experiences was obtained in this experiment, in that No. 2 (splenectomized) developed as severe a jaundice as did the control. A fourth animal (not given in the table) whose splenic vein had been ligated succumbed to an equivalent dose before jaundice developed. At autopsy the finding of new-formed veins was considered to render the experiment equivalent to one on a normal dog.

In another experiment (Table IV) the same results were obtained with a serum of less hemolytic power.

As the transplanted vein was found at autopsy to have been occluded by a comparatively recent thrombus (probably antedating the administration of serum), this experiment must be regarded as including a double test of obstruction to the venous outflow from the spleen. The relatively greater resistance to jaundice of No. 16 as compared with No. 51 may be partly explained by the greater resist-

TABLE III.

Jaundice after Administration of Hemolytic Serum (Serum No. 3, 0.4 Cc. per Kilo).

Time after administration.	Dog 4. Vein switch (2 mos.). 0.45 B. H.-0.325 C. H. Slight anemia.	Dog 2. Splenectomy (2½ mos.). 0.425 B. H.-0.25 C. H. Slight anemia.	Dog 25. Normal control. 0.475 B. H.-0.35 C. H.
Before	Urine normal.	Urine normal.	Urine normal.
1 day after	No bile.	Bile moderate.	Bile trace.
2 days "	" "	" "	" moderate.
4 " "	" "	" "	" absent.
6 " "	" "	" absent.	

TABLE IV.

Jaundice after Administration of Hemolytic Serum (Serum No. 4, 0.2 Cc. per Kilo).

Time after administration.	Dog 16. Vein transplant (occluded vessels) (1 mo.). 0.4 B. H.-0.25 C. H. Anemia present.	Dog 1. Splenectomy (3 mos.). 0.4 B. H.-0.25 C. H. Slight anemia.	Dog 51. Ligation (6 mos.). 0.45 B. H.-0.325 C. H. No anemia.	Dog 27. Normal control. 0.45 B. H.-0.35 C. H.
Before	Urine normal.	Urine normal.	Urine normal.	Urine normal.
1 day after	No bile.	No bile.	No bile.	Bile marked.
2 days "	" "	" "	Bile marked.	" moderate.
3 " "	" "	" "	" "	" trace.
5 " "	" "	" "	" " (Bile still present on the 14th day.)	" absent.

ance of the red cells in the former animal, but the longer duration of jaundice in No. 51 than in the control must be noted as an exception to the general rule.

The results of these experiments, therefore, indicate that the mechanical factor of the method of the blood's approach to the liver is of importance in determining the degree of resistance to hemolytic agents. Our results in this regard are in accord with those obtained by Pepper and Austin (3) on the influence of the site of hemoglobin injections on the production of jaundice.

Duration and Repair of Anemia after Administration of Hemolytic Agents.

Interpretation of the severity and duration of the anemia caused in the various test animals is complicated by several factors. In the first place the original operation has been shown (1) to cause anemia. As the test animals were sometimes given the hemolytic agent while more or less anemic, we have had to analyze our results with constant reference to this factor. Moreover, we have found that in most cases the severity of the anemia is usually the converse of the resistance of the red cells. In some cases, however, animals with the most fragile cells developed the least anemia after administration of hemolytic agents. Changes in weight in the animals of these experiments seem to bear no definite significant relation to the anemia.

We have previously stated (4) that the anemia caused in splenectomized animals by hemolytic agents is, as shown by direct blood examination, of greater severity, runs a longer course, and has a longer period of repair than is the case in the normal dog. By greater severity was meant that the hemoglobin and red cell count reached lower figures than in the control. The actual blood destruction, however, (taken by estimating the drop from the condition immediately before administering hemolytic agents) was usually less in splenectomized than in normal dogs. Our experiments of this year show that the same statements hold true after transplantation of the splenic vein into the vena cava, Eck fistula, and ligation of the splenic veins. The "actual blood destruction" of the test animals has been constantly less than in the normal controls, and in a few instances even the degree of anemia as expressed by the hemoglobin and red cell count was less severe in the test animals. The "greater severity" of the anemia must therefore be considered as open to question, and the "actual blood destruction" in the test animals less than in the normal controls. This, therefore, removes an apparent contradiction in our former work; namely, that although the splenectomized animal showed less signs of blood destruction (jaundice), nevertheless its anemia was more severe than that of the normal control.

The duration of the anemia of the test animals has been longer than that of the normal controls and paralleled that of the splenectomy controls. The difference, however, of both test and splenectomy dogs from the normal controls has been less striking than in our previous work; and like the variable degree of anemia after splenectomy, must be referred to changes in unknown factors (possibly such as diet or season or differences in toxicity of the serum).

The slowness of repair after interference with the splenic function, as contrasted with that in the normal animal is shown in Table V. The red cells show the same temporary decreased resistance to hypotonic salt solution, as has been previously commented upon (3).

In the second experiment, the red cells of No. 68 (Eck fistula) dropped 1,000,000 less than the control, but took 2 months for recovery, as opposed to 19 days of the control. In a third experiment smaller doses of the third series failed materially to affect the blood picture.

A similar experiment with hemolytic immune serum is presented in Table VI.

In a fourth experiment a weaker serum failed to cause anemia in a splenectomized dog as well as in one with occluded splenic vein transplant; however, an animal with ligated splenic vein developed about the same amount of anemia as the control, but took much longer to recover.

Behavior of Leukocytes.

Both toluylenediamin and hemolytic immune serum cause a marked leukocytosis, reaching its height in one to three days and lasting two to four weeks. The first and greatest rise (actual and relative) is in the polymorphonuclears and transitional cells; this is later followed by a less marked and more persistent rise of small lymphocytes and eosinophils. There was no essential difference in the reaction of the test dogs and their controls, except that the latter were still affected by doses too small to affect the former. These changes resemble those described by us in the leukocytes following the various operative procedures on the spleen, and probably indicate a general rather than any specific interference with the leukocytic elements of the blood.

TABLE V.

Duration and Severity of Anemia after Administration of Toluylenediamin (0.34 Gm. per Kilo).

Time after administration.	Dog 71. Splenic vein transplant.			Dog 79. Splenectomy.		
	Hemoglobin.	Red blood cells.	Resistance.	Hemoglobin.	Red blood cells.	Resistance.
	per cent		*B.H. C.H.*	*per cent*		*B.H. C.H.*
Before	98	6,785,000	0.45 –0.325	74	5,720,000	0.4 –0.275
1 day after	102	6,940,000	0.5 –0.3	84	4,770,000	0.5 –0.275
3 days "	58	5,490,000	0.475–0.275	60	4,250,000	0.425–0.275
5 " "	68	5,340,000	0.475–0.325	43	3,380,000	0.45 –0.25
7 " "	72	4,820,000	0.475–0.325	52	3,820,000	0.45 –0.25
9 " "	83	5,070,000	0.5 –0.3	63	4,080,000	0.425–0.25
12 " "	96	5,890,000	0.5 –0.3	66	4,320,000	0.425–0.25
16 " "	98	6,210,000		69	4,530,000	
22 " "	96	6,350,000	0.5 –0.3	68	4,510,000	0.4 –0.275
29 " "				76	5,280,000	0.4 –0.25
35 " "			0.5 –0.3	88	5,590,000	0.4 –0.275
41 " "			0.475–0.325	88	5,480,000	0.4 –0.35
49 " "	98	6,800,000		92	5,680,000	0.4 –0.25

Time after administration.	Dog 51. Ligation.			Dog 75. Normal control.		
	Hemoglobin.	Red blood cells.	Resistance.	Hemoglobin.	Red blood cells.	Resistance.
	per cent		*B.H. C.H.*	*per cent*		*B.H. C.H.*
Before	80	4,920,000	0.45 –0.275	85	6,180,000	0.425–0.275
1 day after	90	4,800,000	0.5 –0.325	96	5,080,000	0.45 –0.35
3 days "				48	4,590,000	0.475–0.275
5 " "	60	3,820,000	0.375–0.25	38	2,980,000	0.45 –0.3
7 " "				45	3,630,000	0.475–0.3
9 " "	62	3,310,000	0.45 –0.3	55	3,930,000	0.45 –0.3
12 " "	65	3,980,000	0.45 –0.275	68	4,800,000	0.475–0.3
16 " "	72	4,320,000		80	5,200,000	0.475–0.3
22 " "	74	4,650,000		85	5,820,000	0.425–0.3
29 " "	76	4,950,000	0.475–0.35			
35 " "	84	5,330,000				
41 " "	78	5,340,000	0.425–0.3			
49 " "	87	5,550,000	0.45 –0.3			

TABLE VI.

Duration and Severity of Anemia after Administration of Hemolytic Serum (0.4 Cc. per Kilo).

Time after administration.	Dog 4. Vein transplant (2 mos.).		Dog 2. Splenectomy (3½ mos.).		Dog 25. Normal control.	
	Hemoglobin.	Red blood cells.	Hemoglobin.	Red blood cells.	Hemoglobin.	Red blood cells.
	per cent		*per cent*		*per cent*	
Before	97	6,960,000	85	4,950,000	103	6,400,000
1 day after	83	4,840,000	78	4,920,000	82	5,040,000
6 days "	65	3,940,000	55	3,670,000	58	3,950,000
10 " "	62	3,750,000	67	3,890,000	78	4,480,000
16 " "	70	4,390,000	67	4,090,000	80	4,880,000
19 " "	90	5,060,000			93	5,490,000
26 " "	96	6,010,000	78	5,260,000	106	6,060,000
31 " "	102	6,400,000	82	5,590,000		

SUMMARY.

1. Dogs whose splenic veins or portal vein (Eck fistula) have been transplanted into the inferior vena cava, or whose splenic veins have been ligated, show a lessened tendency to jaundice similar to that exhibited by splenectomized animals.

2. Although the previously existing anemia and the concomitant increased resistance of the red cells of these animals are undoubtedly factors in the greater resistance to hemolytic agents, the lessened tendency to jaundice is, in part at least, due to a mechanical factor dependent on the course of the blood supply to the liver.

3. The additional anemia caused in the test animals by hemolytic agents is usually less than in the controls, although the total fall from the original normal may be greater. This applies to the splenectomized as well as the other test animals and is a modification of our former statements in regard to the severity of the anemia in splenectomized dogs.

4. Although the destruction of blood in these animals is less than in the normal controls, the repair of the same takes considerably longer than in the controls. This confirms similar results previously obtained in splenectomized animals.

5. The white cells exhibit much the same changes as they do following the administration of hemolytic agents to splenectomized or normal animals. As these changes are not unlike those following uncomplicated splenectomy or the operations here discussed, they cannot be considered as characteristic of any of the above procedures, but perhaps as an accompaniment of any temporary increased blood destruction.

6. The reaction of test and control animals is substantially the same, whether the jaundice is caused by toluylenediamin or hemolytic immune serum.

BIBLIOGRAPHY.

1. Krumbhaar, E. B., Musser, J. H., Jr., and Peet, M. M., The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. XIV. Changes in the Blood Following Diversion of the Splenic Blood from the Liver. A Control Study of the Effects of Splenectomy, *Jour. Exper. Med.*, 1916, xxiii, 87.
2. Pearce, R. M., Austin, J. H., and Krumbhaar, E. B., I. Reactions to Hemolytic Serum at Various Intervals after Splenectomy, *Jour. Exper. Med.*, 1912, xvi, 363. Pearce, R. M., Austin, J. H., and Eisenbrey, A. B., II. The Relation of Hemoglobinemia to Hemoglobinuria and Jaundice in Normal and Splenectomized Animals, *Jour. Exper. Med.*, 1912, xvi, 375. Pearce, R. M., Austin, J. H., and Musser, J. H., Jr., III. The Changes in the Blood Following Splenectomy and Their Relation to the Production of Hemolytic Jaundice, *Jour. Exper. Med.*, 1912, xvi, 758. Pearce, R. M., and Peet, M. M., VII. The Effect of Hemolytic Serum in Splenectomized Dogs, *Jour. Exper. Med.*, 1913, xviii, 494.
3. Austin, J. H., and Pepper, O. H. P., XII. The Importance in the Production of Hemolytic Jaundice of the Path of Hemoglobin to the Liver, *Jour. Exper. Med.*, 1915, xxii, 675.
4. Krumbhaar, E. B., Musser, J. H., Jr., and Pearce, R. M., VIII. Regeneration of the Blood of Splenectomized Dogs after the Administration of Hemolytic Agents, *Jour. Exper. Med.*, 1913, xviii, 665.

A QUANTITATIVE ANALYSIS OF THE INFLUENCE OF THE SIZE OF THE DEFECT ON WOUND HEALING IN THE SKIN OF THE GUINEA PIG.

By KATE C. SPAIN, M.D., and LEO LOEB, M.D.

(*From the Department of Pathology of the Barnard Free Skin and Cancer Hospital, St. Louis.*)

(Received for publication, July 9, 1915.)

The investigations of Loeb, Addison, and Spain have shown that normal as well as regenerative growth of the epidermis of various species of animals differs markedly, that there is a distinct relationship between the structures of the various skins and their proliferative energy, and that the structure of the normal skin and its behavior during regeneration are apparently determined by the proliferative energy of the cells.[1]

We undertook to determine the influence of the size of the wound on the rate of wound healing in the different types of skin, and in this communication we deal with the results obtained in the guinea pig. In one set of animals a thin flap of skin measuring approximately 2 sq. mm. was removed from the ear of each animal; in another set flaps measuring 4 sq. mm. were removed. After 2, 5, 7, 9, 11, and 14 days pieces were cut out in each set for microscopic examination. Three to five pieces were examined at each period, in both the 2 sq. mm. and the 4 sq. mm. series. All the pieces were cut into several sections. The same measurements were made as in the previous investigations. The results are given in Tables I, II, III, and IV.[2]

[1] Loeb, L., and Addison, W. H. F., *Arch. f. Entwcklngsmechn. d. Organ.*, 1911, xxxii, 44. Addison, W. H. F., and Loeb, L., *Arch. f. Entwcklngsmechn. d. Organ.*, 1913, xxxvii, 635. Spain, K. C., *Jour. Exper. Med.*, 1915, xxi, 193.

[2] The microscopic measurements were carried out by Dr. Spain.

TABLE I.

Four Millimeter Series.

Day.	No. of piece.	Length of tongue.	Length of defect.	Length of tongue and defect.	No. of living cell rows and width of stratum germinativum.		
					Tip of tongue.	Insertion of tongue.	Old epithelium.
		mm.	*mm.*	*mm.*			
2		0.498		3.952	1–3	4–5	4
	1	0.454	3.0		(14.6μ)	(47.1–53.8μ)	(41.3μ)
		0.526 } 0.511		3.954	1–3	4–5	4
	2	0.619	2.813		(15.3μ)	(49.53–57.6μ)	(45.3μ)
		0.462		4.086	1–3	4–5	4
	3	0.511	3.113		(16.1μ)	(42.7–56.9μ)	(51.4μ)
3½		0.0823 } 0.894			1–3	5–7	5
	1	0.0965	2.0	3.788	(19.2μ)	(55.2–67.4μ)	(56.9μ)
					Middle of wound.		
5	1	Closed		2.137	7	7–11	7
					(76.3μ)	(69.2–117.3μ)	(64.7μ)
	2	"		2.167	6	7–11	7
					(73.9μ)	(88.4–119.3μ)	(68.7μ)
	3	"		2.772	6	7–11	7
					(69.2μ)	(84.0–126.9μ)	(65.3μ)
7	1	"		1.567	7	5–7	5
					(70.3μ)	(57.2–83.4μ)	(53.8μ)
	2	"		1.662	6	5–7	5
					(62.8μ)	(62.6–93.3μ)	(53.4μ)
	3	"		1.852	6	5–7	5
					(61.7μ)	(61.5–88.1μ)	(56.1μ)
9	1	"		1.514	5	5–7	5
					(58.0μ)	(59.2–79.6μ)	(46.2)
	2	"		1.715	5	5–7	5
					(55.6μ)	(48.5–65.7μ)	(48.4μ)
	3	"		1.562	4	5–7	4
					(41.1μ)	(52.6–64.4μ)	(39.6μ)
11	1	"		1.372	3	5–7	4
					(32.5μ)	(42.3–54.5μ)	(37.3μ)
	2	"		1.309	3	5–7	4
					(29.6μ)	(49.9–67.6μ)	(45.7μ)
	3	"		1.330	3	5–7	4
					(33.8μ)	(50.9–61.3μ)	(38.4μ)
14	1	"		1.228	3	5–7	4
					(26.9μ)	(53.4–60.7μ)	(39.9μ)
	2	"		1.086	3	5–7	4
					(30.7μ)	(48.6–57.4μ)	(34.6μ)
	3	"		1.079	3	5–7	4
					(28.5μ)	(51.9–55.3μ)	(43.0μ)

TABLE II.

Four Millimeter Series.

Day.	No. of piece.	Size of cell.	Size of nucleus.	No. of mitoses in one area 1 mm. long x 50μ wide. New epithelium.		No. of mitoses in one area 1 mm. long x 50μ wide. Old epithelium.	
2	1	μ	μ	0		29	
	2	15.3 x 9.6	11.6 x 7.6	1	1/3	28	29 2/3
	3			0		32	
3½	1						
	2	16.3 x 9.7	11.8 x 7.5	38		30	
	3						
5	1			4		15	
	2	12.1 x 9.7	9.1 x 6.5	7	6 1/3	12	13 2/3
	3			8		14	
7	1			4		15	
	2	12.6 x 9.8	9.3 x 6.9	4	5	16	15 1/3
	3			7		15	
9	1			4		14	
	2	11.9 x 9.6	9.2 x 6.9	5	4 2/3	13	14
	3			5		15	
11	1			4		13	
	2	11.8 x 9.7	8.7 x 6.4	3	3 1/3	12	12
	3			3		11	
14	1			4		13	
	2	11.2 x 9.7	8.9 x 6.1	3	3 1/3	12	12 2/3
	3			3		13	

Changes in the Size of the Wound and Tongue.

The various headings under which the data are arranged in these tables are the same as in the tables in our preceding communications.[3] Although the figures of only three pieces are given in the tables, in the majority of cases additional pieces were examined, and on the whole the results were concordant. In the third column of Tables I and III we find the figures for the length of the tongue in the various periods. Two days after making the wound a tongue

[3] Addison, W. H. F., and Loeb, L., *Arch. f. Entwcklngsmechn. d. Organ.*, 1913, xxxvii, 635. Spain, K. C., *Jour. Exper. Med.*, 1915, xxi, 193.

TABLE III.

Two Millimeter Series.

Day	No. of piece.	Length of tongue.	Length of defect.	Length of tongue and defect.	No. of rows of living cells and width of stratum germinativum. Tip of tongue.	Insertion of tongue.	Old epithelium.
		mm.	*mm.*	*mm.*			
2		0.30		2.03	1–2	3–4	3–4
	1	0.23	1.504		(13.4μ)	(30.6–43.8μ)	(34.7μ)
		0.38 } 0.265		1.956	1–2	3–4	3–4
	2	0.15	1.426		(12.6μ)	(28.7–45.6μ)	(37.8μ)
		0.25		2.23	1–2	3–4	3–4
	3	0.28	1.68		(11.5μ)	(38.4–41.9μ)	(33.6μ)
5		0.538		2.15	3–4	7–11	7–9
	1	0.682	0.83		(38.9μ)	(91.3–115.3μ)	(69.2μ)
		0.513 } 0.6250		2.28	3–4	7–11	7–9
	2	0.787	0.984		(46.9μ)	(90.9–109.6μ)	(66.3μ)
		0.692		1.98	3–4	7–11	7–9
	3	0.511	0.782		(42.3μ)	(88.8–111.6μ)	(71.6μ)
7	1	1.076 } 0.922	0.007	1.852	5–6	7–11	6–7
		0.769			(63.3μ)	(78.0–123.07μ)	(68.5μ)
					Middle of wound.		
	2	Closed		1.662	5–6	7–11	5–6
					(64.2μ)	(78.6–84.7μ)	(58.8μ)
	3	"		1.568	5–6	7–11	5–6
					(62.7μ)	(72.6–89.6μ)	(61.5μ)
9	1	"		1.461	3–4	7–9	4–5
					(46.1μ)	(69.3–78.4μ)	(48.4μ)
	2	"		1.388	3–4	7–9	4–5
					(52.3μ)	(72.3–84.2μ)	(49.9μ)
	3	"		1.569	3–4	7–9	4–5
					(48.2μ)	(60.8–80.7μ)	(52.8μ)
11	1	"		1.204	3–4	5–7	4–5
					(48.2μ)	(50.8–69.2μ)	(44.2μ)
	2	"		1.406	3–4	5–7	4–5
					(41.5μ)	(50.2–64.7μ)	(41.2μ)
	3	"		1.284	3–5	5–6	4–5
					(53.8)μ	(49.7–67.2μ)	(43.1μ)
14	1	"		1.208	3–4	5–7	3–4
					(37.9μ)	(46.1–57.6μ)	(36.4μ)
	2	"		1.197	3–4	5–7	3–4
					(38.4μ)	(42.3–61.5μ)	(44.2μ)
	3	"		1.228	3–4	5–7	3–4
					(42.3μ)	(51.9–63.8μ)	(35.3μ)

TABLE IV.

Two Millimeter Series.

Day.	No. of pieces.	Size of cell.	Size of nucleus.	No. of mitoses in one area 1 mm. long x 50 μ wide.			
				New epithelium.		Old epithelium.	
	1	μ	μ	1		44	
2	2	13.4 x 8.9	9.2 x 7.3	2	$1\frac{1}{3}$	45	44
	3			1		43	
	1			28		26	
5	2	14.6 x 9.9	10.3 x 7.9	26	26	23	24
	3			24		23	
	1	Open					
7	2	14.6 x 9.7	9.8 x 7.6	Open 72		26	
	3	Closed					
		12.1 x 8.6	9.5 x 7.5	Closed 42		20	
	1			27		20	
9	2	13.4 x 8.8	9.5 x.7.2	22	22	16	$17\frac{2}{3}$
	3			17		17	
	1			15		14	
11	2	11.7 x 7.5	9.1 x 6.6	14	$15\frac{1}{3}$	15	$13\frac{2}{3}$
	3			17		12	
	1			11		13	
14	2	11.2 x 7.3	9.3 x 6.7	14	$12\frac{2}{3}$	14	$14\frac{1}{3}$
	3			12		16	

of regenerating epidermis measures 0.265 mm. in the 2 mm. series, and almost twice as much in the 4 mm. series. After three and one-half days the tongue has increased considerably and it is closed after five days in the 4 mm. series. In the 2 mm. series the length of the tongue has enlarged considerably at this period. It is more than double as long as two days after the operation, but the wound is not yet closed. In two pieces the wound was closed after seven days, in one it was still open in the 2 mm. series. After nine days the wound is closed in all the series. The figures in the 3 mm. series, which Addison and Loeb communicated previously,[4] stand generally

[4] Addison, W. H. F., and Loeb, L., *Arch. f. Entwcklngsmechn. d. Organ.*, 1913, xxxvii, 635.

between the figures in the 2 and 4 mm. series. Slight variations which occur are probably due to the fact that in the previous work the exact size of the wound had as yet not been considered as of very great importance and there may therefore have been slight variations in the size of the original wound in the 3 mm. series. We may conclude from these figures that the larger the wound the more rapidly the tongue enlarges and the earlier the closure of the wound takes place, within the variations in size of the wound chosen in our experiments. This difference in the energy of outgrowth of the epithelial tissue is already noticeable within the first two days. Again, from the second to the fifth day the increase in size of the tongue over the size two days after the operation is absolutely greater in the 4 mm. than in the 2 mm. series.

A study of the changes in the size of the defects agrees with the changes in the size of the tongues (Column 4, Tables I and III). Two days after the operation the defect has decreased 1.025 mm. in the 4 mm. series and a little less than half as much (0.461 mm.) in the 2 mm. series. From two days to three and one-half days a further decrease of 0.81 mm. in the size of the defect has taken place in the 4 mm. series. The rate of the decrease is approximately the same as in the preceding period in the 4 mm. series. Sometimes between three and one-half and five days the defect disappears in the 4 mm. series. In the 2 mm. series it decreases 0.639 mm. from the second to the fifth day. Again the defect decreases from the second to the fifth day more in the 4 mm. than in the 2 mm. series. We find, therefore, that the decrease in the size of the defect is in the 4 mm. series very much more rapid than in the 2 mm. series. The figures for the 3 mm. wounds stand between those for 2 and 4 mm.

Contraction of Wound.—In the fifth column of Tables I and III we find the figures for the length of tongues plus defect or of the length of the former defect, after it has been covered by the tongues. The figures in this column after the closure of the wound and the difference between the decrease in the defect and the increase in the tongues in the different periods give an indication of the contraction which takes place in the area of the wound simultaneously with the outgrowth of the tongues.

If we consider first the 4 mm. series, we find no noticeable con-

traction before the end of the second day; between the second and third and a half day there is possibly a very slight contraction. There is a noticeable contraction beginning before the end of the fifth day. A decided contraction, therefore, takes place at the time of the closure of the wound and shortly preceding this period. Between the fifth and seventh day a further decided contraction takes place. Between the seventh and fourteenth day the contraction continues, but at a generally diminishing rate.

In the 2 mm. series the contraction begins much later than in the 4 mm. series, and again at the period of closure and the time preceding it; *viz.*, between the fifth and seventh days. During this period the contraction is quite noticeable. From the seventh to the fourteenth day the contraction continues here with gradually decreasing intensity. In the 4 mm., as well as in the 2 mm. series, the contraction is therefore most marked in the beginning and gradually decreases. At the time of closure the contraction amounts to about 0.4 to 0.5 mm. in both series. Between the ninth and eleventh days the contraction is in both cases somewhat greater than 0.2 mm. Fourteen days after the operation the area of the healed wound has shrunken in both series to about the same area. Therefore, the contraction that takes place in the 4 mm. series is absolutely much greater than that in the 2 mm. series. The amount of contraction in the two series differs, therefore, in the period preceding the seventh day. Between the seventh and fourteenth days the contraction is absolutely approximately the same in both series. The contraction is therefore greater in larger wounds. The approximate contraction in the two series is as follows (Table V).

TABLE V.

Days.	4 mm. series. *mm.*	2 mm. series. *mm.*
0 to 2		
2 to 3½	0.05	
3½ to 5	0.4	
5 to 7	0.68	0.5
7 to 9	0.09	0.22
9 to 11	0.22	0.23
11 to 14	0.14	0.04
Total	1.58	0.99

These figures show that both outgrowing of the tongues and contraction are concerned in the closing of the defect and that both are greater in the larger wound.

Mitoses.—The number of mitoses in the 4 mm. series is given in Table II, and in the 2 mm. series in Table IV. We distinguish between the mitoses in the tongue (here the number of mitoses in one of the tongues over an area 50 μ wide is counted) and in the old epithelium (the number of mitoses in the old epithelium is determined over an area extending 1 mm. from the insertion of the tongue into the old epithelium and 50 μ wide). After the closure of the wound the mitoses in half the epithelium covering the defect are counted.

At two days we find in the 4 mm. series in the tongue $\frac{1}{3}$ of a mitosis in one unit area, in the old epithelium 30 mitoses in a unit area; in the 2 mm. series there are $1\frac{1}{3}$ mitoses in one unit area in the tongue, and 44 mitoses in the old epithelium. As we stated in an earlier communication, at first there are almost no mitoses in the tongue and many mitoses in the old epithelium. In the animals with the smaller wounds there are more mitoses in the old epithelium, although the tongues are greater in the animals with the larger wounds. We may therefore conclude that the tongue formation is not directly dependent on the mitoses but on the migration of the cells. We notice furthermore that also in the tongue the mitoses are more frequent in the smaller wounds. Within a very short time the mitoses increase rapidly in the tongue, and at three and one-half days the mitoses are more frequent in the tongue than in the old epithelium in the 4 mm. series. In the old epithelium the number remains at three and one-half days about the same as at two days.

At five days the wound is closed in the 4 mm. series. We find therefore a sudden decline in the number of mitoses in both former tongue and old epithelium, but the decrease is greater in the tongue, where the pressure of cell layers which meet from opposite directions upon each other is greatest. In the 2 mm. series where the wounds are not yet closed the mitoses in the tongues increase markedly, just as the mitoses did in the tongues in the 4 mm. series at three and one-half days. In the old epithelium, however, no further increase, but a certain decrease takes place, which latter is, however, probably

not very significant and may perhaps be due to a relative diminution in the pull from the tongue.

At seven days the number of mitoses in the former tongue and old epithelium is similar to the number at five days in the 4 mm. series. In the 2 mm. series we must distinguish between those pieces in which a closure of the wound has not yet taken place, and those in which the wound has just closed. In the former the number of mitoses in the tongue increases very much (72), while in the old epithelium the number of mitoses has hardly undergone a change. In the latter directly after the closure a rapid decline in the number of mitoses in the tongues has set in; in these pieces the number of mitoses is about 50 per cent less than in the pieces in which the wound is not yet closed, while in the old epithelium only a relatively much smaller diminution has taken place after the closure (20 mitoses). In a similar way we found in the 3 mm. series a marked rise in the number of mitoses in the tongue and no increase, or rather a slight decline, in the old epithelium just before the time of closure of the wound. In the 4 mm. series the fall in the number of mitoses in the old epithelium directly after the closure of the wound is greater than in the 3 and 2 mm. series. In the latter series in the old epithelium a slight fall had already taken place before the closure of the wound.

At nine days the number of mitoses in the former tongue and old epithelium is about the same as at seven days; in the 4 mm. series there is at the most a very slight decrease. In the 2 mm. series there is a marked decrease in the number of mitoses in the old tongue and a slight decrease in the old epithelium. At both places, however, the number of mitoses is absolutely greater than after the closure of the wound in the 4 mm. series. In all three series the decrease in the number of mitoses after the closure of the wound is greatest in the tongue, while the number of mitoses in the old epithelium is subject to much less marked variations. We see that absolutely the number of mitoses remains higher in the 2 mm. than in the 4 mm. series. Thus the figures for the mitoses at nine days in the former tongues are $4\frac{2}{3}$ and 22 mitoses, and for the old epithelium 14 and $17\frac{2}{3}$ mitoses in the 4 mm. and 2 mm., respectively.

At eleven days there is a further slight decrease in the number of

mitoses in all three series in both the former tongues and the old epithelium; absolutely the decrease is the smaller, the greater the number of mitoses is at nine days.

At fourteen days only at certain places a further very insignificant decrease takes place, while at other places the figures remain almost unchanged. At this time the number of mitoses is approximately the same in all three series, with the exception of the region of the former defect in the 4 mm. series where the number of mitoses is less than elsewhere. The number of mitoses is now approximately the same as in the normal skin. We may conclude that the differences in the rapidity in the outgrowth of the tongues and the resulting differences in the time of closure are mainly responsible for the differences in the variations in the mitoses in the different series. Closure of the wounds is followed by a sudden decline in the number of mitoses, especially in the area of the defect. Therefore the number of mitoses decreases earliest in the 4 mm. series, and absolutely we find there the smallest number of mitoses during the period over which our observations extended. We find therefore a greater number of mitoses in the smaller wound. Of course we have to take into consideration the fact that our observations extend only over an area of the old epithelium 1 mm. long. It is possible that in the 4 mm. series the increase in mitoses extends over a longer area than in the 2 mm. series.

TABLE VI.

No. of Mitoses in the Tongue or in the Epithelium Covering the Former Defect in an Area 1 Mm. Long x 50μ Wide.

Days.		
2	4 mm. series.	$\frac{2}{3}$
	2 " "	5
$3\frac{1}{2}$	4 " "	42
5	4 " "	5.1
	2 " "	40
7	4 " "	6
	2 " "	88
9	4 " "	6
	2 " "	30
11	4 " "	4.7
	2 " "	25
14	4 " "	4.8
	2 " "	21

We have so far considered the number of mitoses in the tongues and in the epithelium covering the former defect independently of the size of the epithelial area. If we reduce all the figures to an epithelial area 1 mm. long, in the same way that we did in the case of the old epithelium, the character of the curve is not changed considerably, but we find in the 2 mm. series the number of mitoses in the region of the old defect exceeding those in the old epithelium and remaining above the normal number for a longer period of time than the figures given above indicate (Table VI).

Changes in the Size of Cell and Nucleus.

The size of the cell and nucleus were measured at the point of insertion of the tongue. Two diameters of cell and of nucleus in the 4 and 2 mm. series are given in Tables II and IV (Columns 3 and 4). If we consider only two diameters, the changes in cell and nucleus are represented in Table VII.

TABLE VII.

	4 mm. series.		2 mm. series.	
Days.	Cell.	Nucleus.	Cell.	Nucleus.
Normal cell	75	49	75	49
2	147	88	119	67
3½	158	88		
5	117	59	145	81
7	124	64	142	75 (open).
			104	71 (closed).
9	114	64	118	68
11	115	56	88	60
14	109	54	81	62

We see that soon after the making of the wound the size of the cell and nucleus increase up to a maximum which is absolutely apparently only a little higher in the 4 mm. than in the 2 mm. series, which, however, is reached much earlier in the 4 mm. than in the 2 mm. series. The maximum is reached just before the closure of the wound in both series. After the closure a sudden decrease in the size of the cells takes place, and from that time on the size remains almost unchanged between the fifth and eleventh day in the 4 mm. series. At fourteen days a very slight decrease is observed, but the

size is still greater than in the normal epidermis. In the 2 mm. series a sudden decline takes place at the seventh day or soon afterwards, and from that time on the size of the cells continues to get smaller at a more rapid rate than in the 4 mm. series. But here also the cell size is still slightly above normal fourteen days after the making of the wound. The changes in the size of the nucleus follow a similar curve; but here the variations are smaller, and the deviations from the normal size not so great as in the case of the whole cell. On the whole, the curves indicating variations in cell and nuclear size, on the one hand, and in the number of mitoses run parallel to each other in the 4 mm. as well as in the 2 mm. series. But the size of cell and nucleus seems to return less quickly to the normal condition than the number of mitoses. Furthermore, the cell size increases more in the 4 mm. series than in the 2 mm. series, while the number of mitoses was more increased in the 2 mm. series.

Number of Rows of Living Cells and Width of Stratum Germinativum.

The last three columns in Tables I and III give the number of rows of living cells and the width of the stratum germinativum, at the tip of the tongue, insertion of tongue, and in the old epithelium near the insertion of the tongue. These figures are not so definite as those of the size of the cells, and in case there is an apparent contradiction between the two sets of figures, the figures for the size of the cells are to be preferred.

We see that at two days the number of cell rows is everywhere greater in the 4 mm. series than in the 2 mm. series, and the thickness of the stratum germinativum is also greater in the 4 mm. series. At three and one-half days the number of cell rows and the thickness of the stratum germinativum increase slightly. At five days the number of cell rows and the thickness of the living epithelium are still greater in both the 2 mm. and 4 mm. series; and the figures are similar in the 2 and 4 mm. series at the place of insertion of the tongues and in the old epithelium. The main difference exists in the epithelium covering the defect. In the 4 mm. series the two tongues have met at that time, exert a pressure on each other, and

we find, therefore, a very much thicker layer and more cell rows in the 4 mm. than in the 2 mm. series at this point. At seven days the figures are very similar to those at five days over the former defect in the 4 mm. series. From then until the fourteenth day a gradual decrease takes place in the thickness of the living epithelium as well as in the number of cell rows in this area.

In the 2 mm. series, on the other hand, we find the maximum in the number of cell rows and thickness of the living epithelium reached at the seventh day, again at the time of the closure of the wound. There the maximum is not so great as in the 4 mm. series. The push with which the two tongues coming from opposite sides meet each other is evidently greater in the 4 mm. series than in the 2 mm. series, and this push of the opposing tongues leads to the increase in the number of living cell rows and thickness of the epithelium in this area. Afterwards the epithelium evidently recedes again somewhat or is cast off. Between the eleventh and fourteenth day the decrease is greater in the 4 mm. series than in the 2 mm. series.

At the same time that the maximum is reached over the former defect, directly after the time of closure, a maximum is also reached at the point of insertion of the tongue and in the old epithelium in the 4 mm. series, while in the 2 mm. series the maximum at these two places is reached before the closure of the wound at the fifth day; from the direction of the old epithelium the push towards the wound extends into the tongue which leads to the subsequent closing. The maximum figures are very similar in the 2 and 4 mm. series. While, however, at the seventh day there is already a distinct diminution in the 4 mm. series, the decrease is less at seven days in the 2 mm. series at these two places; this is in accordance with the fact that at seven days the closure takes place in the 2 mm. series, while at that period the wound has already been closed for some time in the 4 mm. series. Afterwards in both series a gradual decrease takes place at these two areas. At the point of insertion the number of cell rows and the thickness of the epithelium are greater than in the old epithelium in both series, throughout the time of observation. From the eleventh to the fourteenth day the figures at these two places are very similar in the 4 mm. series to those in the 2 mm.

series. We may conclude that in both the 4 and in the 2 mm. series a push of the epithelium takes place in the direction towards the wound, and movements of the epithelium occur not only over the defect, but also in the neighboring epithelium, and that these movements may perhaps proceed in rows from the old epithelium towards the wound, so that a maximum is reached in the old epithelium at a slightly earlier period than over the defect. It thus appears that the primary process in the wound healing consists in movements of the epidermis towards the wound, that these movements are carried out with greater energy if the wound measures 4 mm. than if it measures 2 mm., that the pull of the epithelium calls forth mitotic cell division, and that pressure exerted by epithelial cells upon each other leads to a rapid diminution in the mitotic proliferation.

SUMMARY.

1. The larger the wound, the more rapidly the tongue enlarges and the earlier the closure of the wound takes place. Larger wounds heal, therefore, more quickly than smaller wounds within the variations in the size of the wound chosen in our experiments.

2. Both outgrowing of the tongues and contraction of the wound are concerned in the closing of the wound. A marked contraction sets in in the period preceding the closing of the wound and continues over a longer period with gradually diminishing intensity. The contraction, therefore, sets in earlier in the larger wounds. The contraction is also absolutely greater in the larger wound.

3. During wound healing the mitoses increase first markedly in the old epithelium and only very few mitoses can be found in the outgrowing epithelium during the first two days. Very soon the mitotic proliferation extends to the tongue and the number of proliferating cells may here become greater than in the old epithelium. With the closure of the wound a sudden fall in the number of mitoses takes place in both series. This fall is greatest in the tongue. Throughout the time of observation the number of mitoses is greater in the smaller wound. The fall in the number of mitoses directly after the closure of the wound is more sudden in the 4 mm. than in the 2 mm. series. It is, however, possible that the increase of mitoses extends over a larger area in the 4 mm. series than in the 2 mm. series.

4. It is probable that the difference in the rapidity in the outgrowth of the epidermal tongues and the resulting difference in the time of closure are mainly responsible for the difference in the variations in mitoses in the larger and smaller wounds. The longer the period of time over which the pull of the epithelium extends, the greater is the number of mitoses in this area. Therefore it is greater in the 2 mm. series. Closure of the wound is followed by a sudden decline in the number of mitoses especially in the area of the defect. Therefore the number of mitoses decreases earlier in the 4 mm. series, and we find here the smallest number of mitoses during the whole period of our observation.

5. The size of the epithelial cell and nucleus increases soon after the making of the wound. A maximum is reached in both the larger and smaller wound in the period just preceding the closure of the wound; this maximum is therefore reached earlier in the larger wound. Absolutely the maximal size reached in both kinds of wounds is approximately the same or only a little higher in the larger wounds. After the closure of the wound a sudden decline in the cell size takes place in the larger as well as in the smaller wounds. Then a more gradual decline sets in. Fourteen days after the operation the cells are still larger than in the normal skin. The variations in the size of the nucleus are similar to those in the whole cell, but less marked. The curves of variations in cell and nuclear size follow in both the larger and smaller wounds a curve similar to the variations in the number of mitoses. But the cell size returns more slowly to the normal condition than the number of mitoses.

6. The closure of the wound causes an increase in the number of epithelial rows over the defect. This increase is therefore reached at an earlier period in the larger wound. The increase is greater in the larger wound owing to the greater pressure which the two opposing cell layers exert upon each other in the larger wound. In the old epithelium the maximum in the number of cell rows is apparently reached slightly before the closure of the wounds. It seems that the epithelial movements leading to the closure of the wound start in the old epithelium and extend wave-like towards the wound.

7. It thus appears that the primary process in the wound healing consists in movements of the epidermis towards the wound, that

these movements are carried out with greater energy in the case of the larger wounds, that the pull of the epithelium calls forth mitotic cell division, and that pressure exerted by epithelial cells upon each other leads to a rapid diminution in the mitotic proliferation.

INTESTINAL OBSTRUCTION.

V. Proteose Intoxication.

By G. H. WHIPPLE, M.D., F. H. RODENBAUGH, M.D., and
A. R. KILGORE, M.D.

(*From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco.*)

(Received for publication, July 21, 1915.)

In earlier publications Whipple, Stone, and Bernheim (1, 2, 3, 4) have established facts which have an important bearing on this question of proteose intoxication. Some of these points will be reviewed, but for the detailed experiments the original papers should be consulted. In this work closed loops of intestine have been used because it has been established by previous experiments that an acute intoxication develops, and the toxic substance may be obtained in concentrated form in the material which accumulates in the closed loops. The intoxication noted in simple intestinal obstruction is similar, but usually less intense than that observed in dogs with closed or drained duodenal loops. Much evidence has been furnished to demonstrate that the essential toxic substance is very similar or even identical in obstruction and in closed intestinal loops.

All recent work speaks strongly for the theory of a definite intoxication in intestinal obstruction and closed loops of intestine. The doubts expressed by Hartwell and Hoguet (5) have been answered by Draper (6). Hartwell and Hoguet claimed that loss of fluid and consequent dehydration of the tissues were primary and the intoxication a secondary result of this dehydration. Whipple, Stone, and Bernheim have claimed that the intoxication was primary and the dehydration of the tissues secondary to the intoxication. Draper has been able to show that a dog can be dehydrated in four days by pilocarpin to the same degree encountered in duodenal obstruction. One of us has been able to bring about a similar dehydration and blood concentration by means of pilocarpin and vigorous salt purgation

over a period of thirty-six hours. The dogs which are dehydrated by means of drugs show no evidence of intoxication and recover their weight very promptly when given abundant food and water. It is safe to assume that there is a primary intoxication in intestinal obstruction and with closed intestinal loops.

When a loop of small intestine is washed out, closed by tapes, and a gastro-enterostomy made to reestablish the continuity of the intestine, we may attribute any resultant intoxication to one or both of two factors: (1) bacteria, and (2) intestinal mucosa. We have shown elsewhere that destruction of the mucosa prevents the formation of any toxic substance in the loop; in other words, the mucosa is essential to the production of the poison. Whether bacteria are essential or not is more difficult of proof and this point has not been established as yet beyond criticism. Murphy and Brooks (7) submit an experiment which suggests that bacteria are essential, but it may be claimed that the mucosa of a sterile drained loop, probably in a dog more or less strongly immunized, might not react in the usual manner. The poison formed in this inoculated loop did not give the usual reaction when given intravenously in a normal dog.

It has been claimed by Whipple, Stone, and Bernheim that the poison can be formed in a closed loop with no demonstrable lesions in the mucosa. This has been disputed by Hartwell and Hoguet (5, 8), and by Murphy and Brooks (7). It may be admitted that commonly the fluid accumulates in the closed loops, that this causes pressure and interference with the blood supply and finally injury to the mucosa. Some cases, however, are found in which only a little pasty material is found in a closed loop immediately after death. We wish to emphasize the fact that in such cases immediately after death we are unable to find the slightest histological evidence of injury to the mucosa. It is not necessary to find histological evidences of abnormality to support our belief that this mucous membrane is abnormal in that it forms a toxic proteose and allows its escape into the blood stream. We are convinced that these cells can present an abnormal physiological activity without any demonstrable histological change.

Absorption of this poison from the intestinal tract brings up some points of much practical importance. It is probable that injury of the mucosa will greatly facilitate toxic absorption, but it must be

remembered that the normal mucosa can not absorb any of the poison. Further, it is known that closed loops may be filled with much toxic material without giving any increase in intoxication. Dogs whose loops are filled with a lethal dose of toxin will live as long as control dogs whose loops are left empty at the end of the operation. Drained loops which are washed frequently give ample evidence of intoxication. This shows that much, if not the greater part of the intoxication is due to absorption from the mucous membrane alone rather than from the material in the lumen of the gut. Our attention should be concentrated on the mucous membrane in an effort to discover the fundamental change which is responsible for the toxin production and the reaction which may be responsible for its sudden return to normal activity.

The chemical nature of this poison is of great interest and of some practical importance. When its identity is established it will be possible to study all similar intoxications with the possibility of discovering the best means of combating the intoxication or neutralizing the poison or helping in its elimination. The fundamental objective of course is to check the formation of the poison at its source, but this may be impossible.

It is a very elusive substance and of necessity concealed in a mass of confusing material. Most workers in this field have hazarded a guess as to the identity of the poison. Albeck (9) believed that it belonged in the group of putrefactive poisons. Murphy and Brooks (7) believe that it may be similar to the substance sepsin isolated by Faust (10). It has been suggested that it might resemble the β-iminazolyl ethylamine of Dale (11). Some of the reactions of this closed loop poison resemble those produced by the anaphylatoxin.

As evidence for its chemical analysis and identification it is to be recalled that this substance is not capable of sensitizing guinea pigs for the characteristic anaphylactic reaction. Dogs can be immunized to the poison, but this protection is not great, and an immune animal can scarcely withstand a double lethal dose. The immunity does not reside in the body fluids which are inert toward the poison. The immune organs or the autolyzed tissue juices contain ferments which destroy the poison *in vitro* (Whipple, Stone, and Bernheim (3)).

It is to be recalled that this poison may be thrown out of a rich

albuminous solution by boiling. The poison is more or less completely removed by Berkefeld filters (Murphy and Vincent (12) and Kukula (13)). Our earlier experiments show that autolysis with normal intestinal mucosa for a week will not destroy the poison (2). Long autolysis with mucosa weakens its toxic power and after many months (8 to 12) the poison may be completely destroyed. This resistance to digestive enzymes is characteristic and of considerable importance.

EXPERIMENTAL OBSERVATIONS.

The experiments given below show that the poison is not destroyed by pancreatic digestion for seven days. The most important observation, that alcohol precipitates this poison, is of great help in the isolation of this substance. Five volumes of 95 per cent alcohol will cause complete precipitation of the poison, and the filtrate is free from the toxic substance. This point has been established beyond a doubt by repeated experiments, and in this way a great number of confusing substances may be eliminated. Further purification may be effected by solution of the alcoholic precipitate and reprecipitation by one-half saturation with ammonium sulphate. Dialysis may be used, as the toxin does not pass a collodion membrane. The pure substance is a primary proteose, and may be classed as a heteroproteose because it resists digestion. It is very toxic, and 100 mg. may be sufficient to poison a fifteen pound dog with the characteristic fatal reaction.

Methods.

Dogs were used in all the experiments. The kymograph observations were taken from the carotid artery exposed by a small incision at the root of the neck. In the abdominal operations all the usual aseptic precautions were observed. Ether anesthesia was used in all experiments. Hammarsten's method for testing the urine for proteoses may be outlined as follows. Equal parts of urine and saturated ammonium sulphate are heated to a boil. The precipitate is thrown down in a centrifuge, the supernatant fluid decanted, and the precipitate washed repeatedly with alcohol to remove the urobilin. The residue is dissolved in water, boiled, and filtered to remove albumin. The solution is extracted with chloroform and finally tested with the biuret.

Poison Not Destroyed by Complete Pancreatic Digestion.

Duodenal loop fluid (30 cc., a known lethal dose) was put away in the incubator at 38° C. after mixing with a freshly prepared dog's pancreas. The pancreas had been ground with sand, allowed to stand for 1 to 2 hours mixed with the loop fluid, made alkaline to litmus, and diluted to a thin soup. The mixture was protected from putrefaction by chloroform and toluol. Digestion proceeded for 7 days. There was no unpleasant odor, and the mixture was almost wholly fluid. It was passed through a thick Gooch crucible filter giving a clear amber fluid which was used to inject into a normal dog (Dog S-16).

Dog S-16.—Small adult mongrel, male; weight 14¼ pounds.

Feb. 17, 3 p.m. Intravenous injection of the clear filtrate just described (30 cc.). This gave the usual reaction seen after the injection of the toxic material into dogs. The initial fall in blood pressure was transient with rapid return to normal. The progressive lasting fall in pressure began at the end of 30 minutes. 3.30 p.m. Dog is shocked and shows air hunger. 8.00 p.m. Death with extremely low temperature.

Autopsy.—The findings are typical of death by a large dose of the poison found in closed duodenal loops. Splanchnic engorgement and fluid in the intestines are the striking features.

Poison Precipitated by Alcohol and Soluble in Water.

Duodenal loop fluid (40 cc.), precipitated by three volumes of 95 per cent alcohol and heated for an hour over a boiling water bath, was filtered.

1. The filtrate or alcoholic extract was evaporated slowly in a vacuum at 80° C. to a small volume and tested on a normal dog with negative results. Kymograph tracings taken during the injection showed a prompt initial fall in blood pressure with return to normal in one or two minutes. The substance which causes the initial fall in blood pressure similar to the reaction of tissue extracts is soluble in alcohol. The dog recovered without showing the least signs of intoxication.

2. The precipitate was extracted with ether for several days and the ether extract allowed to evaporate, giving a fatty residue. This ether residue was taken up in alcohol and tested on a normal dog with negative result.

3. The precipitate (extracted by alcohol and ether) was finally extracted with water over a water bath, and the watery filtrate obtained by rapid paper filtration. It was tested on Dog S-39. Fatal poisoning in three and one-half hours.

4. The residue was finally digested with intestinal mucosa effecting a complete solution. This fluid was tested on a normal dog with negative results.

Dog S–39.—Small yellow mongrel, male; weight 13¼ pounds.

Apr. 19, 11 a.m. Intravenous injection of watery extract of the alcoholic precipitate from the duodenal loop fluid. This caused some initial drop in blood pressure, followed by a return to normal lasting during the kymograph observa-

tion. 1.30 p.m. Dog is gravely shocked; much diarrhea and vomiting. 2.30 p.m. Death in collapse. Rectal temperature 30° C.

Autopsy.—The findings are typical of acute poisoning with duodenal loop fluid. Splanchnic congestion very marked. Blood concentration is striking; dry weight 26.3 per cent.

Alcoholic Precipitate Contains Poison.

Duodenal loop fluid (30 cc.) was allowed to digest with a little intestinal mucosa plus chloroform for over two weeks. This gave a clear broth-like filtrate which contains the poison in such experiments. This clear solution was treated with five volumes of 95 per cent alcohol, and the mixture was allowed to stand for three days after being brought to a boil over a water bath. A very scanty precipitate came down on standing. This precipitate was collected on a small filter and washed with alcohol and ether. It was estimated, by comparison with fibrinogen precipitates collected in similar manner, at 30 to 60 mg. This precipitate was completely dissolved in water and used for intravenous injection as before.

Dog O-74.—Small mongrel, female; weight 8½ pounds.

Apr. 21, 2.30 p.m. Intravenous injection of the above preparation caused no initial drop in blood pressure, but a slight secondary fall after a period of 30 minutes. 4 p.m. Dog vomits repeatedly. 5 p.m. Dog vomits repeatedly; salivation and diarrhea marked. 10 p.m. Death.

Autopsy.—Typical picture of acute poisoning by duodenal loop fluid.

The preceding experiments (Dogs S-39 and O-74) show that this poison can be obtained from a mixed solution by alcoholic precipitation. The precipitate when extracted with water yields all of the toxic substances, and the amount of the poison must be very small indeed.

Duodenal Loop Fluid—Toxic Proteose.

Loop fluid (1,100 cc.) was collected from six dogs. All these dogs had closed loops of varying length including the duodenum and upper part of the jejunum, varying from 20 to 30 inches in length. The loops were isolated by means of tapes which occluded the lumen of the intestine, and a gastro-enterostomy was performed a few inches below the lower ligature. This mixture was preserved a few days with chloroform and toluol at room temperature. It was then incubated for 48 hours at 38° C. and again allowed to stand at room temperature for 2 weeks.

1,000 cc. of the mixture were precipitated with 5 liters of 95 per cent alcohol at room temperature and allowed to stand at room temperature for many weeks with occasional shaking.

100 cc. were tested by preliminary experiments to determine the amount of toxic substance and some of its reactions. The fluid was boiled over a free flame giving a moderately voluminous precipitate. The supernatant fluid was poured

off and the coagulum extracted again with boiling water which was added to the decanted fluid, making about 120 cc. in all. This fluid was filtered through paper. It reacted faintly alkaline to litmus. Half saturation with ammonium sulphate gave a moderate flocculent precipitate. Five volumes of 95 per cent alcohol gave about the same amount of precipitation. Complete saturation with ammonium sulphate gave but a little more precipitate than did half saturation. Saturation with sodium chloride gave less precipitate than alcohol or ammonium sulphate.

Dog 15-21.—Normal adult spaniel, female; weight 23 pounds.

Mar. 31, 11 a.m. Intravenous injection of 100 cc. of the clear duodenal loop fluid which had been tested above. Temperature at end of the injection 99.4° F. 12 m. Dog not intoxicated, vomited little mucus. No feces passed. Rectal temperature 99.7°F. 12.30 p.m. Dog is vomiting again and passed one semisolid stool; pulse is fair. 1.30 p.m. Dog is vomiting again but is not severely shocked. 3 p.m. Dog is quite sick. Pulse is of a low tension. Dog vomited bile-stained mucus. Temperature 102.5° F. Next day dog appeared normal.

This experiment shows that one-eleventh of the entire fluid did not contain a lethal dose of poison and probably contained about one-half or less than one-half of a lethal dose. We may assume with some justification that the remaining 1,000 cc. of duodenal loop fluid contained from 3 to 6 times a lethal dose for a dog of 15 to 20 pounds' weight.

Duodenal loop fluid (1,000 cc.) was precipitated with 5 liters of 95 per cent alcohol, standing at room temperature for 12 weeks. The precipitate was not abundant. It was thrown upon a paper filter and partially dried. The precipitate was then washed from the filter paper with about 550 cc. of hot distilled water. The mixture made faintly acid to litmus with acetic acid and boiled over the free flame gave an abundant flocculent precipitate which was removed by centrifugalization. The supernatant fluid was poured through a paper filter, giving a total amount of 350 cc. of pale canary yellow, slightly opalescent fluid.

Of this clear filtrate 250 cc. were precipitated with an equal volume of saturated solution of ammonium sulphate and allowed to stand at room temperature. The abundant precipitate appeared slowly and was finally thrown down by the centrifuge. The supernatant fluid was decanted and the precipitate dried between filter paper. This material was dissolved in water to about 125 cc. and heated to boiling over a free flame. It gave a fine flocculent precipitate which was thrown down in the centrifuge, and amounted to about 0.25 the volume of the original ammonium sulphate precipitate. This represents almost the last trace of albumin. The supernatant fluid is almost water-clear and is faintly acid to litmus, but it gives a good precipitate on half saturation of ammonium sulphate. The volume of this solution equals 115 cc., and 65 cc. of this fluid given intravenously in Dog 15-50 cause fatal intoxication in 3½ hours (see also Dog 15-51).

Dog 15-50.—Small black and tan, adult, male; weight 13 pounds.

June 11, 12 m. Kymograph observation with ether anesthesia. Purified loop fluid (65 cc.), described above, given intravenously. Blood pressure was very little influenced by this injection, but the heart was somewhat slowed and respira-

tion was considerably accelerated. Muscular twitchings were quite conspicuous at the end of the injection and this was due probably to the ammonium sulphate remaining in this solution. 12.45 p.m. Dog was removed from kymograph and out of ether. Passed one soft stool and vomited once. 2.30 p.m. No vomiting, but considerable salivation; pulse is weak and dog is cold and looks badly shocked. 3.15 p.m. Blood pressure very low; no vomiting and no diarrhea. Dog in semi-stupor and very cold. 3.45 p.m. Condition the same; temperature 96.8° F. 4 p.m. Death with autopsy immediately.

Autopsy.—Blood examination showed a considerable excess of antithrombin, as is usual after injection of the crude duodenal material; thorax and the lungs are normal; liver is deep purple and remarkably engorged, otherwise normal. Spleen is greatly enlarged and the splanchnic vessels are very conspicuous. Stomach shows a pale pylorus and cardia and a deep pinkish mucosa in the middle zone. Mucus and fluid very abundant in stomach and small intestine. Duodenum shows a deep purple red velvety mucosa coated with large amounts of mucus and contains much fluid. This color fades to a pink color in the ileum. The colon shows a mottled pinkish red mucosa. This picture is typical of acute poisoning by a large dose of duodenal loop fluid or fluid obtained from above an intestinal obstruction.

The remaining purified duodenal fluid (50 cc. in amount) which had been precipitated with alcohol and then with ammonium sulphate, dissolved in water, boiled, and filtered, was next dialyzed against 0.8 per cent sodium chloride in a collodion tube. This gave a turbidity and slight increase in volume. After dialysis for 18 hours the fluid was boiled and a small precipitate removed by the centrifuge. Filtration through paper gave an odorless, water-clear, but slightly opalescent fluid which was used for further tests. Dialyzed fluid (5 cc.) was precipitated in an Esbach tube with Tsuchiya's reagent (phosphotungstic acid 1.5, hydrochloric acid 5, alcohol 100 parts). This gave 1 gm. per 1,000 cc., as read from the tubes. This represents about 1 mg. per cc. of fluid. The dried weight was estimated carefully for 2 cc. and equalled 1.04 per cent. Nitrogen determination showed that the ammonium sulphate had not been completely removed by dialysis. The dried weight by calculation, allowing for 0.8 per cent sodium chloride, gives a maximum of 120 mg. of material in 50 cc. of this solution. The Esbach determination gives 50 mg. per 50 cc. The first estimation by the dried weight is probably too high, but more accurate than the Esbach. A fairly safe estimate of 100 mg. of proteose in this 50 cc. of fluid may be made. This fluid (50 cc.) was injected into Dog 15–51 with fatal result.

Dog 15–51.—Small fox-terrier, female; weight 15½ pounds.

June 14, 12 m. Kymograph observation with ether anesthesia. Solution of pure proteose (50 cc.) from duodenal loop fluid given intravenously. This caused no reaction on the blood pressure or pulse beat. 12.30 p.m. Animal seems quite unaffected by injection. Removed from kymograph. 1.30 p.m. Dog recovered and seems normal; walks about cage. 4 p.m. Dog is prostrated and greatly shocked; slow deep respiration, vomiting, and diarrhea noted. Temperature 104.2° F. Pulse tension poor. 10 p.m. Dog appears shocked and cold.

June 15, 9 a.m. Dog found dead and cold; much fluid feces in cage.

Autopsy.—Thorax, heart, and lungs normal; spleen and liver only moderately congested; stomach contains blood-tinged fluid and shows moderate congestion of the mucosa. Duodenum contains a good deal of mucus and fluid, and the mucosa is pinkish red and definitely engorged with blood. Jejunum and ileum show a similar picture and contain much watery fluid with mucus. This picture is characteristic of moderately acute intoxication and this dog was given just about a minimal lethal dose of the poison, causing death in about 15 hours with characteristic delayed symptoms of shock, associated with vomiting and diarrhea. It is probable that this dog received approximately 100 mg. of purified proteose obtained from duodenal loop fluid.

This isolation of a primary proteose from the duodenal loop fluid is well established by the above experiments. One liter of duodenal loop fluid contained several lethal doses of the characteristic poison. The toxic substance was first precipitated by five volumes of 95 per cent alcohol and the precipitate dissolved in water. This solution made faintly acid and boiled gave an abundant albuminous precipitate which was removed. The solution was treated with equal parts of a saturated solution of ammonium sulphate which gave a white flocculent precipitate. This precipitate was dissolved in water and the solution again boiled to remove all albumin. This clear filtrate contained the characteristic poison, and 65 cc. poisoned a dog fatally in four hours (Dog 15–50). The rest of this clear filtrate was dialyzed for eighteen hours, again boiled and filtered, giving a water-clear, slightly opalescent fluid. This fluid (50 cc.) contained about 100 mg. of proteose and was sufficient to poison fatally a dog weighing fifteen pounds (Dog 15–51).

This method of isolation removes practically all substances from the duodenal loop fluid mixture with the exception of the primary proteoses. This pure substance gives the identical toxic symptoms noted after injection of the crude duodenal loop fluid. Moreover, the alcoholic extract or filtrate is non-toxic and the bulk of the toxic material can be isolated and purified without great loss. This is strong, if not conclusive, evidence that the essential toxic substance has been isolated from the duodenal loop fluid and that the toxic substance is a primary proteose.

DISCUSSION.

Many interesting points concerning proteose intoxication have been brought out by the work of Chittenden (14) and his coworkers. These investigators show that certain proteoses may stop urinary secretion without much blood pressure reaction. This toxic proteose of intestinal obstruction may cause a sudden stoppage in urinary secretion and no primary blood pressure effect. The fall in blood pressure may not appear until hours after the injection when the symptoms of shock are in evidence.

Some proteoses may be changed before their excretion in the urine,—a primary proteose being excreted as a secondary proteose. It is very important to emphasize that when these proteoses escape from the body they do so by way of the kidneys. It has been shown by Hartwell and Hoguet (5) and others that administration of fluid is of benefit in the intoxication of intestinal obstruction, and it is highly probable that the diuresis aids in the elimination of the poison which may escape in part by way of the urine.

We have attempted to isolate this toxic proteose from the urine of dogs with intestinal obstruction and closed loops of intestine, and following injection of the toxic proteose. There are obvious difficulties in this work when we consider the small amount of the poison (100 mg.) which may produce fatal intoxication. Its injection in considerable amount is attended by cessation of renal excretion. It is not sufficient to state that a proteose appears in the urine after the intravenous injection of this toxic proteose or after an experimental closed intestinal loop. Various procedures may cause albuminuria in a dog, and this is often associated with a definite proteosuria (as shown by Hammarsten's method) without any evidences of intoxication. The determination of a proteose in small amounts in a dog's urine has no special significance. We have attempted to isolate a toxic proteose from dog's urine after obstruction or closed intestinal loops, but so far without success. It is possible that this specific primary proteose may be modified in some way before its excretion in the urine.

Animals react differently to the proteose groups of poisons. Underhill (15) and others point out that dogs are susceptible to pro-

teose intoxication, whereas cats and rabbits are very resistant. Davis and Morgan (16) have shown that cats are very resistant to the poison found in the closed intestinal loops of dogs or cats. Moreover, cats will survive a closed intestinal loop of a certain type much longer than a dog under parallel conditions.

Dogs react constantly to this proteose obtained from closed intestinal loops. The intensity of the clinical symptoms of salivation, vomiting, diarrhea, and prostration depend upon the amount of poison injected. The blood may show a great increase in antithrombin, which will delay or completely prevent clotting *in vitro*. This reaction is variable, but usually follows intravenous injection of a variety of proteoses. In fatal poisoning the splanchnic engorgement is the striking feature. The liver and spleen are swollen and purple. The mucosa of the duodenum especially, but also of the whole intestine, is greatly congested and may be a velvety purplish red color.

It is to be emphasized that other poisons can cause this poisoning with peculiar splanchnic paralysis and engorgement in dogs. It has been noted with various putrefactive poisons, toxic bases or amines (Faust), also after large doses of adrenalin, and in fatal anaphylaxis. It is not safe to draw conclusions about the nature of a given poison solely from its physiological action, but chemical tests must be added.

An interesting experiment has been performed by Murphy and Brooks (7), who isolated the gall bladder after introducing diluted intestinal contents. The dogs died with symptoms of intoxication, and the contents of their gall bladders were toxic to normal dogs, giving the usual picture of proteose intoxication. It is possible that growth of bacteria in association with the mucosa of the gall bladder can produce a toxic proteose. More work must be done, however, to establish this important point. On the other hand, it is possible that some putrefactive poison is responsible, because such poisons and toxic proteoses give similar reactions when given intravenously in dogs.

It is to be emphasized that the group of putrefactive poisons is not found in closed intestinal loops, and the only toxic element is a primary proteose.

Proteose intoxication causes a striking rise in the incoagulable nitrogen of the blood, which may double in amount in a period of three hours. Intestinal obstruction or a closed intestinal loop may cause a great rise in the non-coagulable blood nitrogen, which may even rise to ten times normal. This seems to depend within limits upon the severity and acuteness of the intoxication. Detailed experiments will be published in the near future, and the incoagulable nitrogen may prove to be of clinical value from the standpoint of diagnosis and prognosis.

SUMMARY.

A definite intoxication develops as a result of a closed intestinal loop and toxic material accumulates in the closed loops. Much evidence has been submitted to show that this loop poison causes the intoxication observed after producing a closed intestinal loop. Sufficient evidence has been presented to prove that the essential poison is present in these closed intestinal loops, and usually in concentrated form.

Chemical study of the contents of closed intestinal loops shows that a single substance or group of substances possesses toxic properties. This resists autolysis and pancreatic and ereptic digestion. It is thrown out of solution by five volumes of alcohol or by half saturation with ammonium sulphate. It is readily soluble in water and is not injured by boiling. It is not removed by dialysis. The method of isolation excludes practically all substances except primary proteoses. The characteristic resistance to digestive enzymes suggests a heteroproteose.

Proteose intoxication in dogs gives a picture identical with that described after poisoning with intestinal loop fluid: early salivation and vomiting, followed by diarrhea and prostration, fall in temperature and blood pressure, and finally death in collapse. Autopsy shows essentially a splanchnic paralysis and remarkable engorgement of liver and spleen, but especially of the mucosa of the duodenum and small intestine. The blood shows great concentration due to loss of fluid and may remain incoagulable because of an excess production of antithrombin.

Proteoses escaping from the blood are excreted in the urine. This toxic proteose concerned in intestinal obstruction has not yet been isolated in the urine, but may be excreted by the kidneys. This probably explains the clinical improvement and lessened intoxication noted after transfusion.

Experimental evidence points to a primary proteose as the essential poison concerned in the intoxication of closed intestinal loops and intestinal obstruction.

BIBLIOGRAPHY.

1. Whipple, G. H., Stone, H. B., and Bernheim, B. M., *Jour. Exper. Med.*, 1913, xvii, 286.
2. Whipple, G. H., Stone, H. B., and Bernheim, B. M., *Jour. Exper. Med.*, 1913, xvii, 307.
3. Whipple, G. H., Stone, H. B., and Bernheim, B. M., *Jour. Exper. Med.*, 1914, xix, 144.
4. Whipple, G. H., Stone, H. B., and Bernheim, B. M., *Jour. Exper. Med.*, 1914, xix, 166.
5. Hartwell, J. A., and Hoguet, J. P., *Jour. Am. Med. Assn.*, 1912, lix, 82.
6. Draper, J. W., *Jour. Am. Med. Assn.*, 1914, lxiii, 1079.
7. Murphy, F. T., and Brooks, B., *Arch. Int. Med.*, 1915, xv, 392.
8. Hartwell, J. A., Hoguet, J. P., and Beekman, F., *Arch. Int. Med.*, 1914, xiii, 701.
9. Albeck, V., *Arch. f. klin. Chir.*, 1902, lxv, 569.
10. Faust, E. S., *Arch. f. exper. Path. u. Pharmakol.*, 1904, li, 248.
11. Dale, H. H., and Laidlaw, P. P., *Jour. Physiol.*, 1910–11, xli, 318.
12. Murphy, F. T., and Vincent, B., *Boston Med. and Surg. Jour.*, 1911, clxv, 684.
13. Kukula, *Arch. f. klin. Chir.*, 1901, lxiii, 773.
14. Chittenden, R. H., Mendel, L. B., and Henderson, Y., *Am. Jour. Physiol.*, 1898–99, ii, 142.
15. Underhill, F. P., *Am. Jour. Physiol.*, 1913, ix, 345.
16. Davis, D. M., and Morgan, H. S., *Bull. Johns Hopkins Hosp.*, 1914, xxv, 39.

ICTERUS.

A Rapid Change of Hemoglobin to Bile Pigment in the Pleural and Peritoneal Cavities.

By C. W. HOOPER, M.D., and G. H. WHIPPLE, M.D.

(*From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco.*)

(Received for publication, July 23, 1915.)

In an earlier communication (1) we have been able to show that bile pigment could be formed from hemoglobin without the agency of the liver. Solutions of hemoglobin were introduced into the blood vessels of dogs whose livers had been excluded from any part in this reaction. There was a prompt formation of bile pigment from hemoglobin with no possible direct liver action. This transformation can take place within a space of two hours when active circulation is maintained in the head and thorax alone. It seemed probable that the endothelium might be the tissue whose activity was responsible for this change of hemoglobin to bile pigments.

This work has received confirmation from experiments of McNee (2) who repeated the experiments of Minkowski and Naunyn with geese. He found that icterus did develop without liver activity and found evidences of endothelial activity. He suggests (3) that the Kupffer cells may normally transform hemoglobin to bile pigments.

The experiments given below prove conclusively that still other tissues can rapidly transform the hemoglobin pigment into bile pigment. There can be no question of any direct liver activity in these experiments. Some transformation may take place within eight hours, but the pigment production in twenty-four hours can usually be estimated with considerable accuracy. This mesothelium of the serous cavities can not effect this transformation as promptly as it is effected in the circulating blood, but the contact with the living pleural cells is not as active and intimate,—not a circulatory contact.

If we assume that the capillary endothelium can transform hemoglobin to bile pigment, it is at once obvious how intimate is the contact of the circulating hemoglobin solution with the living protoplasm of the vessels. The solution of hemoglobin in the serous cavities may be in more or less motion (respiration, peristalsis, or body movements), but at best it is not in very intimate contact with the living protoplasm. The wonder is that the transformation is so prompt and easily recognizable. It is impossible to estimate the relative activity of endothelium and mesothelium, but there can be little difference if we allow for the rapidity of circulation and surface contact.

It has been claimed by older workers (Virchow (4) and many others) that blood standing long in contact with living tissues was slowly changed to a golden pigment hematoidin which is chemically equivalent to bilirubin. Virchow used the term hemolytic icterus for this reason. Guillain and Troisier (5) noted in human cases with pleural hematomas that bile pigment was formed after a considerable period with no complicating liver abnormality. Von der Bergh and Snapper (6) were able to find greater amounts of bile pigment in serous exudates than in the blood serum, and they speak in favor of the extrahepatic formation of bile pigment. That the serous cavities can rapidly transform hemoglobin to bile pigment has not been hitherto recognized.

EXPERIMENTAL OBSERVATIONS.

The experiments are not all given in detail, but the essential facts are given in Tables I and II. A typical experiment from each group is given in sufficient detail, and it is to be emphasized that the majority of these experiments caused no inflammation or even permanent injury to the pleural or peritoneal cavity. In a few cases due to slips in technique there developed a pleurisy or a peritonitis, but these complications did not modify the transformation of hemoglobin to bile pigment. The crystalline hemoglobin causes a slight irritation in the pleural or peritoneal cavity, and it is possible that the inflammatory reaction may assist in the transformation, but it is not an essential factor.

Method.

Active and vigorous dogs were used in all of our experiments. The urine was obtained by catheter, and in every case it was examined before the experiment. The Huppert-Salkowski test for bile pigments was used in examining the urine, and unless otherwise stated 20 cc. of urine were employed in the test. Either fresh or crystalline dog hemoglobin was used. The fresh hemoglobin was obtained by laking washed erythrocytes with distilled water.

The preparation of crystalline hemoglobin was carried out according to the method of Bradley and Sansum (7). The red blood cells obtained from defibrinated dog blood by centrifugalization were washed eighteen or twenty times with normal salt solution, then they were laked with toluene and a little distilled water. The toluene layer was removed and the solution centrifugalized and decanted from the stroma material. This hemoglobin solution was then mixed with 20 per cent by volume of toluene, shaken thoroughly, and set aside in the cold. After twenty-four or forty-eight hours the mixture is nearly a solid mass of large well formed crystals. The crystals are collected by centrifugalization, washed with cold water, spread thin on glass, and dried in a stream of warmed air. Fresh 0.6 per cent salt solution was used as a diluting medium for the fresh or crystalline dog hemoglobin.

The fluids were introduced into or withdrawn from the pleural or peritoneal cavity through a trocar. After withdrawing the pleural or peritoneal fluids they were centrifugalized immediately, the supernatant fluid was decanted and made definitely alkaline by the addition of a saturated solution of sodium carbonate, then a 10 per cent solution of calcium chloride was added until precipitation was complete. After centrifugalization at high speed the supernatant fluid was again poured off, and the calcium bile pigment compound remained as a yellowish precipitate. The precipitate was washed free from other coloring matter and collected again by centrifugalization. Finally the precipitate dissolved in a hot solution of 5 per cent hydrochloric acid in 95 per cent alcohol gave the characteristic blue green color when bile pigments were present.

The method used to determine the amounts of bile pigments quantitatively will be published in the near future. This method in abstract consists of reading the blue green color of the acid alcohol extract in a fixed dilution against a permanent wedge of similar color which has been standardized against chemically pure bilirubin solutions of known amounts. The common sulphonephthalein colorimeter may be used to advantage.

Pleural Experiments.

Bile Pigments Formed from Hemoglobin in Periods of 8 to 66 Hours.

Dog 15–11.—8 and 24 hours. (Table I.) Short haired mongrel, male; weight 14 pounds.

Mar. 17. Dog is normal. 9.30 a.m. 20 cc. of concentrated urine are negative for bile pigments. 10 a.m. 20 cc. of washed, laked red blood cells are introduced into the right pleural cavity with 500 cc. of sterile 0.6 per cent salt solution. 11.10 a.m. No respiratory embarrassment. 2 p.m. 20 cc. of urine are negative for bile pigments and hemoglobin. 6 p.m. 100 cc. of dark red fluid removed from right pleural cavity are negative for bile pigments. 20 cc. of urine are negative for bile pigments and hemoglobin.

Mar. 18, 9.35 a.m. Dog is very active. Rectal temperature 39.1° C. 20 cc. of urine negative for bile pigments and hemoglobin. 10 a.m. 158 cc. of dark red fluid recovered from right pleural cavity are positive for bile pigments. 11.20 a.m. Right pleural cavity irrigated with 500 cc. of 0.6 per cent salt solution. Appetite is fairly good.

Mar. 19, 9 a.m. Dog is listless. Rectal temperature 40.2° C. Respiratory movements over right thorax are impaired.

Mar. 20, 10 a.m. Rectal temperature 39.1° C. Dog is very active and eats well. Respiration normal.

Dog 15–34.—8 hours. (Table I.) Mongrel setter, male; weight 55 pounds.

May 12. Dog is active and vigorous. 8.30 a.m. 20 cc. of urine are negative for bile pigments. 8.45 a.m. 500 cc. of water are introduced into the stomach through a stomach tube. 9.30 a.m. 30 cc. of washed, laked red blood cells are introduced into the right pleural cavity with 1,500 cc. of sterile 0.6 per cent salt solution. 11 a.m. 20 cc. of urine are negative for bile pigments and hemoglobin. 5 p.m. 20 cc. of urine are negative for bile pigments and hemoglobin. 5.30 p.m. 1,175 cc. of dark red, turbid fluid recovered from right pleural cavity. Chemical test is positive (suspicious) for bile pigments. 5.50 p.m. Right pleural cavity is irrigated with 500 cc. of 0.6 per cent salt solution. 6 p.m. Drinks some milk.

May 13, 9.30 a.m. Dog is listless and refuses to eat. Rectal temperature 102.3° F. Respiratory movements over right thorax are impaired, and a friction rub is palpable.

May 14, 10 p.m. Very active. Rectal temperature 101.8° F. Respiratory movements are normal.

Dog 15–39.—17 and 66 hours. (Table I.) Black mongrel, female; weight 32 pounds.

May 10. Dog is in excellent condition. 9.30 a.m. 20 cc. of concentrated urine are negative for bile pigments. 11.15 a.m. 30 cc. of washed, laked red blood cells suspended in 1,100 cc. of sterile 0.6 per cent salt solution are introduced into the right pleural cavity. 5.20 p.m. 930 cc. of dark red fluid, recovered from the right pleural cavity, give a positive (suspicious) test for bile pigments. 20 cc. of urine are negative for bile pigments and hemoglobin. 5.30 p.m. Right pleural cavity is irrigated with 500 cc. of 0.6 per cent salt solution. 6 p.m. Respiratory movements over right thorax are somewhat delayed.

May 11. Dog is active and vigorous. Rectal temperature 102.6° F. Respiratory movements are normal.

May 17. Dog is in excellent condition. Weight 32 pounds. 3.30 p.m. 20 cc. of urine are negative for bile pigments. 5 p.m. 30 cc. of washed, laked red blood cells are introduced into the left pleural cavity with 1,000 cc. of 0.6 per cent salt solution. 6.10 p.m. 20 cc. of urine are negative for bile pigments and hemoglobin. Respiratory movements are somewhat delayed over left thorax.

May 18, 9.30 a.m. Rectal temperature 103.2° F. Respiratory movements are markedly delayed over left thorax. 20 cc. of urine are negative for bile pigments and hemoglobin. 10 a.m. 390 cc. of dark red fluid recovered from the left pleural cavity contain 0.14 mg. of bile pigment. 5.30 p.m. Animal is quite active.

May 19. Dog is not very lively. 9 a.m. Rectal temperature 102.8 °F.

May 25. Weight 32 pounds. Dog is active and vigorous. Rectal temperature 102.2° F. 4.30 p.m. 20 cc. of urine are negative for bile pigments. 4.45 p.m. 500 cc. of water are introduced into the stomach through a stomach tube. 4.50 p.m. The right pleural cavity is irrigated with 500 cc. of sterile 0.6 per cent salt solution. The fluid recovered is water-clear; bile pigment test negative. 6.10 p.m. 10 gm. of crystalline dog hemoglobin dissolved in 1,100 cc. of 0.6 per cent salt solution are introduced into the right pleural cavity. 6 p.m. Respiratory distress is not marked.

May 26. Dog is quite active and eats well. The movements of the right thorax are somewhat impaired. Rectal temperature 101.8° F. 2.10 p.m. 20 cc. of urine are negative for bile pigments and hemoglobin.

May 27. Rectal temperature 102.2° F. Movements of the right thorax are markedly impaired. Refuses food. 12.35 p.m. 20 cc. of urine are faintly positive for bile pigments.

May 28. Rectal temperature 102.8° F. 10.30 p.m. 20 cc. of urine are faintly positive for bile pigments. 11 a.m. 510 cc. of dark red turbid fluid recovered from the right pleural cavity contain 5.40 mg. of bile pigments. 11.20 a.m. Right pleural cavity is irrigated with 500 cc. of sterile 0.6 per cent salt solution.

May 29. Dog is normal.

Dog 15–52.—24 hours. (Table I.) Bull terrier mongrel, male; weight 41.5 pounds.

June 16. Dog is normal. 10 p.m. 20 cc. of urine are negative for bile pigments. 11.35 a.m. 9 gm. of crystalline dog hemoglobin dissolved in 1,000 cc. of 0.6 per cent salt solution are injected into the right pleural cavity. 12 m. Respiratory distress is not marked. 6 p.m. Animal drinks some milk.

June 17. Dog is quite active. Respiratory movements over right thorax are impaired. Rectal temperature 102.8° F. 10 a.m. 20 cc. of urine are negative for bile pigments. 11.30 a.m. 575 cc. of dark red, turbid fluid recovered from the right pleural cavity contain 0.18 mg. of bile pigment. 5 p.m. Respiratory movements are quite normal.

June 18. Dog is very active. Rectal temperature 102.4° F.

TABLE I.

Hemoglobin Changed to Bile Pigment in Pleural Cavity.

Dog No. Date.	Weight.	Pleural cavity.	Time in pleural cavity.	Bile pigment tests.	Bile pigments.	Fluid introduced.	Fluid recovered.	Bile in urine. Before.	Bile in urine. After.	Remarks.
1915	*lbs.*		*hrs.*		*mg.*	*cc.*	*cc.*			
15–11 Mar. 17	14.0	Right.	8	0	0	620	100	0	0	Fresh dog hb.
15–11* Mar. 17	14.0	“	24	+	–	620	158	0	0	“ “ “
15–34 May 12	55.0	“	8	(?)+	–	1,580	1,175	0	0	“ “ “
15–39 May 10	32.0	“	7	(?)+	–	1,160	930	0	0	“ “ “
15–40 May 10	22.0	“	6	(?)	–	700	555	0	0	“ “ “
15–41 May 12	36.0	“	8	(?)+	–	1,480	1,230	0	0	“ “ “
15–39 May 17	32.0	Left.	17	+++	0.14	1,105	390	0	0	“ “ “
15–40 May 17	21.5	“	18	+++	0.09	805	265	0	0	“ “ “
15–52 June 16	41.5	Right.	24	++++	0.18	1,000	575	0	0	Crystalline dog hb., 10 gm.
15–54 June 16	36.5	“	24	++	–	800	395	0	0	“ “ “ 7 “
15–53 June 16	29.5	“	25	+	–	800	408	0	0	“ “ “ 7 “
15–56 June 16	39.0	“	25	++	–	900	318	0	0	“ “ “ 7 “
15–41 May 20	36.5	Left.	43	+++	0.15	805	254	0	+ (faint)	Fresh “ “
15–43 May 20	34.0	Right.	44	++++	0.36	1,100	808	0	0	Crystalline “ “ 4 “
15–40 May 25	22.0	“	65	++++	3.60	940	430	0	+ (faint)	Fresh “ “
15–39* May 25	32.0	“	66	++++	5.40	1,100	510	0	+ (faint)	Crystalline “ “ 10 “

* See history for details.

Dog 15–43.—43 hours. (Table I.) Mongrel spaniel, male; weight 34 pounds.

May 20. Dog is in excellent condition. Rectal temperature 102° F. 12.10 p.m. 20 cc. of urine are negative for bile pigments. 12.15 p.m. 500 cc. of water are introduced into the stomach through a stomach tube. 3.10 p.m. 4 gm. of crystalline dog hemoglobin dissolved in 1,100 cc. of sterile 0.6 per cent salt solution are introduced into the right pleural cavity. 5.30 p.m. 20 cc. of urine are negative for bile pigments and hemoglobin.

May 21. Dog is active and eats well. Respiratory movements over the right thorax are impaired. Rectal temperature 102.2° F.

May 22. Rectal temperature 103.1° F. 10.30 a.m. 20 cc. of urine are negative for bile pigments and hemoglobin. 11 a.m. 808 cc. of dark red, turbid fluid recovered from the right pleural cavity contain 0.36 mg. of bile pigments.

May 23. Rectal temperature 101.8° F. Dog is normal.

The preceding experiments are conclusive proof that mesothelium can rapidly transform hemoglobin into bile pigment. The lining cells of the pleural cavity like other body cells (probably endothelium) can form bile pigments out of hemoglobin, either freshly laked or crystallized. This solution of hemoglobin is not in very intimate contact, not in circulatory contact, as is hemoglobin in contact with the endothelium of the capillaries, yet the transformation takes place promptly in a few hours. There is experimental evidence of this bile pigment transformation in the pleura within eight hours, but a very definite amount is formed within eighteen to twenty-four hours, an amount often sufficient for accurate determination.

Dog 15–20.—8 hours. (Table II.) Mongrel poodle, male; weight 19.5 pounds.

Mar. 29. Dog is normal. 10 a.m. 20 cc. of concentrated urine are negative for bile pigments. 10.30 a.m. 35 cc. of washed, laked red blood cells are introduced into the peritoneal cavity with 800 cc. of 0.6 per cent salt solution. 12.30 a.m. 20 cc. of urine are negative for bile pigments and hemoglobin. 2 p.m. Rectal temperature 101.3° F. 5.30 p.m. 20 cc. of urine are negative for bile pigments and hemoglobin. 6.30 p.m. 300 cc. of dark red turbid fluid recovered from the peritoneal cavity give a questionably positive test for bile pigments. 6.45 p.m. Peritoneal cavity is irrigated with 2,000 cc. of 0.6 per cent salt solution. Urine gives positive (suspicious) test for bile pigments.

Mar. 30. Dog is very lively and eats well. Rectal temperature 101.7° F.

Dog 15–43.—28 hours. (Table II.) Mongrel spaniel, male; weight 33.5 pounds.

June 17. Dog is in excellent condition. 9.45 a.m. 20 cc. of urine are negative for bile pigments. 11.15 a.m. 7 gm. of crystalline dog hemoglobin dissolved in 2,800 cc. of 0.6 per cent salt solution, introduced into the peritoneal cavity.

12 m. Dog shows no embarrassment. 5.30 p.m. Animal listless and refuses to eat. Considerable rigidity of abdomen.

June 18. Rectal temperature 103.8° F. Animal is listless, will not eat, and lies quietly in cage. Abdominal muscles are very tense. 2 p.m. 20 cc. of urine are positive for bile pigments. 3.20 p.m. 755 cc. of dark red turbid fluid recovered from the peritoneal cavity give a positive test for bile pigments. 3.35 p.m. Peritoneal cavity irrigated with 3,000 cc. of sterile 0.6 per cent salt solution. 5 p.m. Rectal temperature 104.2° F.

June 19. 9 a.m. Animal is found dead. Autopsy showed a diffuse fibrinous peritonitis. Other organs are negative.

Dog 15–55.—48 hours. (Table II.) Mongrel hound, male; weight 40.5 pounds.

June 16. Dog is in excellent condition. 2 p.m. 20 cc. of urine are negative for bile pigments. 2.40 p.m. 7 gm. of crystalline dog hemoglobin dissolved in 3,000 cc. of 0.6 per cent salt solution introduced into the peritoneal cavity. 6 p.m. Dog is quite active. 20 cc. of urine are negative for bile pigments and hemoglobin.

June 17. Rectal temperature 102.2° F. 10.10 a.m. 20 cc. of urine are positive for bile pigments. Hemoglobin negative.

June 18. Dog is very lively and eats well. 9.30 a.m. Rectal temperature 103.6° F. 1.30 p.m. 20 cc. of urine are negative for bile pigments and hemoglobin. 2.30 p.m. 310 cc. of dark red, turbid fluid recovered from peritoneal cavity contain 0.27 mg. of bile pigment. 2.50 p.m. Peritoneal cavity irrigated with 2,000 cc. of 0.6 per cent salt solution. 5.30 p.m. Dog is very active.

June 30. Dog is normal.

TABLE II.

Hemoglobin Changed to Bile Pigment in Peritoneal Cavity.

Dog No. Date.	Weight.	Time in peritoneal cavity.	Bile pigment tests.	Bile pigments.	Fluid introduced.	Fluid recovered.	Bile in urine. Before.	Bile in urine. After.	Remarks.
1915	*lbs.*	*hrs.*		*mg.*	*cc.*	*cc.*			
15–20 Mar. 29	19.5	8	(?)	—	985	300	0	(?)+	Fresh dog hb.
15–43 June 17	33.5	28	+	—	2,800	755	0	+	Crystalline dog hb., 7 gm.
15–34 May 20	56.0	44	++++	0.25	4,000	1,360	0	(?)+	" " " 7 "
15–41 June 17	36.5	48	++++	0.18	3,000	310	0	+	" " " 7 "
15–57 June 16	24.5	48	++++	0.32	2,500	255	0	+	" " " 7 "
15–55 June 16	40.5	46	++++	0.27	3,000	310	0	+	" " " 7 "

The preceding experiments and Table II show that the peritoneal cavity can transform hemoglobin into bile pigment with the same promptness as the pleural cavity. The two cavities are lined by a similar mesothelium, so there need be no surprise when the reactions are found to be similar. The difference in absorption from the two cavities is obvious. The pleural cavity gives slow absorption, most of the fluid introduced is recovered, and little if any of the formed bile pigment appears in the urine. The peritoneal cavity gives a pretty rapid absorption, most of the fluid introduced is taken up, and the bile pigments appear in the urine as a result.

DISCUSSION.

A difference in absorption from the pleural and peritoneal cavities has been noted and it comes out clearly in Tables I and II. The relatively rapid absorption from the peritoneal cavity causes the escape of bile pigments in the urine. Even in the pleural experiments, however, there is considerable absorption which indicates that in both pleural and peritoneal cavities there is an outflow of fluid. Whether there is interchange between the introduced fluid and the body fluids is uncertain, but much interchange is unlikely. In these experiments the dogs were all normal and their urine free from bile pigment at the beginning of the experiments, which means that the serum was negative to bile pigments by the test employed. That the blood pigments could have been absorbed into the blood, changed to bile pigments in association with the liver, and again diffused into the pleural fluid, is inconceivable. The observations in the pleural experiments (Table I) show that the urine was free from hemoglobin and bile pigments during the first forty-eight hours.

A theoretical objection could be raised to the experiments with the hemoglobin solutions in the peritoneal cavity. It could be argued that this solution comes in contact with the liver and could be modified in this manner. The solution does come in contact with the serous covering of the liver, but not with the hepatic epithelium. Diffusion through the serous epithelium of the liver is conceivable, but most improbable, and the pleural experiments are not open to such an objection.

The use of crystalline hemoglobin seems to cause more irritation of the serous surfaces than does a fresh solution of hemoglobin obtained from freshly laked red corpuscles. When this irritant action lasts over a period of three days there is undoubtedly some new formation of capillaries and proliferation of mesothelium, as well as an escape of various wandering cells. This reaction brings other extrahepatic factors into the equation and apparently accelerates the formation of bile pigment (Table I).

All this evidence indicates that the function of changing hemoglobin to bile pigment is not limited to any single cell or even to two types of cell. It may well be a function of endothelium and mesothelium as well as of hepatic epithelium. Perhaps wandering cells have this property of transforming blood to bile pigment. Many of these wandering cells probably have their origin from endothelium, and may they not retain this functional capacity? It is quite possible that other epithelium besides liver epithelium may be able to effect this transformation. More experiments are being carried out with this point in view. If we go a step further it may be suggested that living protoplasm in general can change hemoglobin to bile pigments.

Still other questions may be raised. Can tissue juices or ferments bring about this transformation of hemoglobin to bile pigment? We have performed many experiments with negative results, but have not yet given up the attempt although success seems unlikely.

CONCLUSIONS.

It is known that hemoglobin can be rapidly changed to bile pigment in a circulation confined to the head, neck, and thorax. This excludes direct liver participation (1).

These experiments show that hemoglobin can be changed to bile pigment within the pleural or peritoneal cavities.

This transformation can usually be detected after eight hours, and the amount can often be estimated quantitatively after an interval of twenty-four hours.

Such experiments demonstrate the importance which may attach to extrahepatic bile pigment formation. That bile pigments can be formed without direct liver activity is established beyond doubt.

It is highly probable that endothelium as well as mesothelium (serous cavities) can transform hemoglobin into bile pigment. It is possible that this property may reside in cell protoplasm in general.

BIBLIOGRAPHY.

1. Whipple, G. H., and Hooper, C. W., *Jour. Exper. Med.*, 1913, xvii, 612.
2. McNee, J. W., *Med. Klin.*, 1913, ix, 1125.
3. McNee, J. W., *Jour. Path. and Bacteriol.*, 1913–14, xviii, 325.
4. Virchow, R., *Virchows Arch. f. path. Anat.*, 1847, i, 379.
5. Guillain, G., and Troisier, J., *Rev. de. med.*, 1909, xxix, 465.
6. von der Bergh, A. A. H., and Snapper, J., *Berl. klin. Wchnschr.*, 1914, li, 1109.
7. Bradley, H. C., and Sansum, W. D., *Jour. Biol. Chem.*, 1914, xviii, 497.

THE PATHOLOGICAL EFFECTS OF ATMOSPHERES RICH IN OXYGEN.

By HOWARD T. KARSNER, M.D.

(*From the Nutrition Laboratory of the Carnegie Institution of Washington, Boston, the Laboratories of Pathology of the Harvard Medical School, Boston, and the School of Medicine of Western Reserve University, Cleveland.*)

Plates 20 to 23.

(Received for publication, September 30, 1915.)

Studies of the pathological effects of oxygen administered under various conditions have not been so numerous or exhaustive as the investigations of its chemical and physiological relations. The fact that oxygen is being used freely as a therapeutic agent, as a prophylaxis against asphyxia in anesthesia, as a protective agent in certain industries, in some forms of deep sea diving apparatus, and in high ascents into the air, makes the study one of practical importance. Even today there is much difference of opinion as to how far oxygen plays a part in the production of some of the manifestations of caisson-disease. Furthermore, oxygen is used extensively in respiration chambers, such as those of the Carnegie Nutrition Laboratory, but the percentage of oxygen in the atmosphere of the chamber is rarely that of out-door air; *i.e.*, 21 per cent, yet it is rarely below 18 or above 24 per cent. The present study deals with the effects on the organism of high oxygen partial pressures (80 to 96 per cent) under ordinary barometric pressure, particularly from the point of view of pathological anatomy and histology. The brief extracts from the literature are taken from the papers that bear directly on the problem in hand.

According to Schmiedehausen (1), Lavoisier shortly after his discovery of oxygen in the atmosphere demonstrated that pure oxygen produces congestion and even inflammation of the lungs. With the introduction of ether and chloroform, the use of oxygen to combat asphyxia began and in later times deep sea diving and the use of oxygen in various industries turned the attention of physiologists and pathologists to studies of the effects of oxygen inhalation. Paul Bert

(2) emphasized the effects on the nervous system of high atmospheric pressures, but apparently overlooked the effects on the lungs. In contrast to this, Lorraine Smith (3) pointed out the inflammatory reaction in the lungs of mice and also called attention to the appearance of moderate congestion in the abdominal viscera. Smith claims, however, that fibrin and leukocytes play no part in inflammation. He found that 40 per cent oxygen for 8 days did not produce pneumonia, but that 80 per cent killed 2 mice in 2 days, while 2 others survived unharmed. An average pressure of 125.3 per cent oxygen killed mice in an average of 64 hours; 180 per cent oxygen killed in 24 hours; and 300 per cent oxygen produced pneumonia in 5 hours. Heller, Mager, and von Schrötter (4), in their extensive studies, ignore pneumonia as a factor in caisson-disease, but Hill and Macleod (5) confirm the findings of Lorraine Smith in regard to pneumonia; they agree, however, with Paul Bert and von Schrötter that in caisson-disease the liberation of compressed gases in the tissues is the most important pathological effect. Hill and Macleod call attention to a considerable variation in individual resistance to oxygen poisoning, "but the larger animals seem just as susceptible as mice." They also state that in regard to production of pneumonia their animals were somewhat more resistant than those of Lorraine Smith.

Schmiedehausen (6) in a series of experiments with 13 animals (2 mice, 3 rabbits, 8 guinea pigs) used pure oxygen by tracheal cannula in 2 animals and high percentages of oxygen in chambers for the rest. His pathological findings are not critically described, but he finds in a general way hyperemia, more or less marked atelectasis, focalized edema, and inflammatory processes. He lays much stress on minor changes after short exposures, but demonstrates no true pneumonia in less than 69 hours' exposure. Schmidt and David (7) caution against the too free or prolonged use of oxygen in chloroform anesthesia. They state that percentages of oxygen as low as 40 to 60 may produce inflammatory changes in the lungs after 70 hours' exposure. David (8) confirms the work of Schmiedehausen and suggests the use of high oxygen partial pressures for the purpose of inducing a pulmonary hyperemia for therapeutic purposes. Bornstein and Stroink (9), working principally with rats under increased barometric pressure, in order to determine the advisability of the use of oxygen by divers, state that high pressures (5 atmospheres of oxygen) for periods as short as 2 to 4 hours produce alveolar edema, desquamation, and slight hemorrhage. They call attention to the fact that the lung findings are similar to those of slightly irritating gases, such as ether, and state that in their animals no organs except the lungs show changes. They state that lower pressures of one to three atmospheres for 20 to 48 minutes are harmless. Schmidt and David (10) regard Bornstein and Stroink's work as confirmatory of their own.

The purpose of the present study is to examine the thoracic and abdominal viscera, as well as the hematopoietic system, in an effort

to explain the more obvious results in the lungs. There is included also a critical study of the pathological histology of the lung changes in comparison with the lungs of control animals. Throughout the study the most careful attention has been given controls, instead of placing any great reliance on the theoretical condition called normal. As will be seen from the reports on different organs, this precaution, particularly in studies on the rabbit, is of extreme importance.

For the purpose of the study the animals were placed in the small animal apparatus devised especially for this investigation by Benedict (11). A supply of carrots sufficient for three days was placed in the chamber and replenished in those cases where the experiments were considerably prolonged. No animal at autopsy showed an empty stomach. Water was supplied freely at all times. Brünig (12) claims that the effects reported by Bornstein and Stroink (13) are due to excessive dryness of the atmosphere rather than its rich oxygen content,[1] but the Benedict apparatus provides for moistening the air.

As the study progressed the details of the examinations varied somewhat. In a few of the earlier experiments animals from stock were placed in the chamber and autopsies performed as soon after death as possible, or the animals were removed from the chamber on exhibition of marked dyspnea, killed by a blow, and the autopsy was performed. It was soon considered advisable to examine the urine before exposure in the chamber and to exclude from the study all animals with albuminuria. The examinations increased in complexity, however. Each animal before entering the chamber was examined for albuminuria, complete blood counts were made, clotting time was determined, hemoglobin estimated, and the percentage of reticulated erythrocytes estimated. The animals that were removed alive from the chamber were examined in the same way, and in addition a sufficient amount of blood was removed from the femoral artery to permit determination of the erythrocyte resistance to hypotonic salt solutions. In spite of the fact that frequently examinations for

[1] Bornstein later wrote to the *Deutsch. med. Wchnschr.* (1912, xxxviii, 2035) maintaining that his controls were sufficient to overcome the objection made by Brünig, to the effect that dryness of the atmosphere in the oxygen chamber produced the pneumonia.

albumin were made on two successive days preceding the exposure to high oxygen, the autopsy on several occasions showed chronic nephritis. This matter will be discussed later.

Twenty-one of the 55 rabbits studied were control animals. Seven of these were in room air in a cage the same size as the special chamber and under the same conditions as to food as those in the chamber. The other controls were in the chamber, with oxygen kept at a low percentage; 6 animals at 21 per cent, 2 at 23 per cent, 1 at 27 per cent, 2 at 28 per cent, and 2 at 31 per cent. One control animal was taken directly from stock.

The pathological examination includes gross and microscopical examination of the heart, lungs, liver, kidney, adrenal, spleen, lymph nodes, and bone marrow, as well as gross examinations of the aorta, stomach, and intestines. Blocks were placed in Zenker fluid and in 10 per cent formalin solution. The routine stain was hemalum and eosin, and in addition the lung sections were stained with the Mallory connective tissue stain for fibrin; numerous frozen sections of the heart, kidney, and liver were stained with Scharlach R for fat.

The pathological findings were at first grouped according to finer grades of difference in oxygen percentage; namely, 70 to 75 per cent, 76 to 80 per cent, 81 to 85 per cent, etc. It was found, however, that these minor changes in percentage were not of apparent importance and that a grouping of 60 to 80 per cent and 80 per cent and higher gave results that were easily distinguishable. The following description of organs is confined to animals which had been exposed for varying periods of time to atmospheres containing 80 per cent and more oxygen. Further studies of lower percentages are now in progress. As will be seen, the examination of these organs is constantly compared with that of the control animals.

Heart.

The examination of the hearts of 19 control animals showed numerous departures from the normal, particularly in the presence of fat in the muscle, foci of chronic interstitial myocarditis, chronic fibrous pericarditis, and in 2 of the 19 cases slight dilatation.

Hearts of Animals in High Oxygen Partial Pressure.—The results of the examination of 26 hearts in this series can be condensed into

the following statements, with the reservation that the changes noted in the control hearts were also found in the oxygen hearts in practically the same proportions.

First, of 9 animals that died in the oxygen chamber the hearts of 7 showed notable dilatation either of the right or of both sides. Two other animals that survived respectively 2 and 5 days' exposure to high oxygen atmospheres also showed bilateral cardiac dilatation. Second, as exposure to high oxygen becomes prolonged, cloudy swelling, meaning loss of transverse striations and coarse granulation in the protoplasm of the fiber cell, becomes practically uniform. Several cases of acute non-suppurative interstitial myocarditis are found in the hearts of high oxygen animals, but these are not sufficiently frequent to be of significance. Of 16 examinations for fat in the high oxygen animals 14 resulted positively as compared with 7 in 11 normal animals,—a difference probably within the margin of error of biological experimentation, but possibly associated with the practically constant cloudy swelling noted above. Litten, Naunyn, Nasaroff, Werhovsky, and Welch have shown that exposure of animals for several days to a temperature of 40° C. results in fatty degeneration of the heart muscle (14). In none of our experiments did the environmental temperature rise above 25° C.

Aorta.

The aortas of 24 animals were examined grossly and arteriosclerosis was found in 3 cases, all of which were controls. It can therefore be stated that no notable effect is produced in the aorta.

Lungs.

Grossly the lungs of the so called normal animals have the normal color and consistence, and crepitate throughout. Occasionally areas of slight congestion are observed posteriorly at the base of one or both lungs (3 animals). One animal showed marked distention of the lung, and a few minute subpleural hemorrhages.

Microscopically the picture with three exceptions is practically constant. The larger blood vessels exhibit wide open lumina containing blood corpuscles; in some of the smaller vessels the lumina

contain fine acidophilic granules, probably the precipitated protein of the serum. The bronchi are of normal size and contain a small amount of granular precipitate, which does not stain for fibrin, but which immediately overlies the epithelium. The epithelium shows well marked granulation of the protoplasm and in many cases contains mucin. Desquamation of epithelium is frequently seen and occasionally there occur small mononuclear cells, probably lymphocytes, in the lumina of the bronchi. In small foci the alveoli are distended and the walls thin, but the lungs for the most part show well marked capillary congestion, even to a point where the capillaries appear as small bullæ projecting into the alveolar spaces. Leukocytes are numerous in the capillaries and in some cases form a striking part of the picture; they sometimes appear within the alveoli. The alveolar epithelium is normal except that occasional desquamated cells showing cloudy swelling are seen. Alveolar edema cannot be demonstrated. In three animals the alveoli are seen to be markedly distended, but the bronchi remain the same as in the other lungs. Throughout the series small lymph nodes are found in relation to the bronchi, made up principally of small lymphocytes with a few large mononuclears included.

Lungs of Animals in High Oxygen Partial Pressure for Approximately Twenty-Four Hours.—These 6 lung specimens show little material departure from the normal except that congestion is slightly more marked (Fig. 1, Rabbit 10). Leukocytes are apparently not more numerous than in many normal lungs. Cloudy swelling and desquamation of alveolar epithelium are seen in all these lungs, and in one lung (Rabbit 13) there is a small area of consolidation made up largely of desquamated alveolar epithelium, which has fused in places to form giant cells. The bronchi show no material changes, but the lymph nodes are enlarged and contain many large mononuclear cells.

High Oxygen for Approximately Forty-Eight Hours.—Of the 9 lungs studied in this series, 5 (Nos. 19, 29, 30, 44, 54) show no more change than do the lungs of the preceding group. Rabbit 18 shows in addition a small focus of desquamative bronchopneumonia similar to that seen in Rabbit 13, but showing more fibrin and leukocytes. In Rabbits 20 and 21 alveolar and bronchial desquamation are marked and numerous alveoli contain a small amount of fine granular acidophilic precipitate in addition to a moderate deposit of fibrin strands. In Rabbit 4, which died in the oxygen chamber, the edema and fibrin are richer, occasional alveoli being almost filled with fibrin. Numerous polymorphonuclear leukocytes appear in the alveoli. As has been already indicated, these differences cannot be accounted for by minor differences in oxygen percentage.

High Oxygen for Approximately Three Days.—Of the 6 animals forming this series the lungs of all save one show serious alterations. In Rabbit 23 intense congestion is present, but the changes are not more marked than those pictured in Fig. 1. All the others show somewhat varying degrees of edema, epithelial desquamation, exudate of leukocytes, and fibrin formation,—in other words, varying degrees of pneumonia. Of the 5 lungs showing this inflammation only one shows complete filling of the alveoli, and in this case the edema occupies most of the space. The bronchi show cloudy swelling of the epithelium, but little mucin formation; there is infiltration of polymorphonuclear leukocytes into the wall and into the lumen; there is marked epithelial desquamation and some of the bronchi show fibrin formation and an occasional erythrocyte. The peribronchial and perivascular lymphatics are frequently the seat of edema. The blood vessels of all kinds and sizes are considerably distended. The alterations in the alveoli are shown in Figs. 2, 3, and 4. They consist of cloudy swelling, and occasionally fatty degeneration of the attached epithelium, desquamation, advanced cloudy swelling, and necrosis; there is moderate infiltration of polymorphonuclear leukocytes and lymphocytes; edema appears as a fine acidophilic precipitate, occupying usually only small areas in the alveoli, but in one case (Rabbit 49) filling the alveolar spaces; fibrils, nodules, and networks of fibrin appear usually in irregularly disposed foci, occupying sometimes a small part, sometimes all of an alveolar space. The fibrin has the typical histology, is acidophilic, and takes the red stain by the Mallory connective tissue method.

High Oxygen for Approximately Four Days.—Of the 4 animals comprising this series one (Rabbit 56) shows only slight changes in the lungs, two (Rabbits 1 and 2) show exudation as seen in most of the lungs of the preceding series, and one (Rabbit 5) shows marked edema in addition to the inflammatory exudate and is similar to the lung of Rabbit 49 of the preceding series. It can safely be said that exposures of approximately 4 days produce practically the same alterations in the lungs as are seen at the end of 3 days.

Rabbit 6 survived 4 days and 20 hours in 83 per cent oxygen. Grossly the lungs were mottled with small areas of congestion. Histologically the lungs show the same marked congestion, epithelial desquamation and degeneration, leukocyte infiltration, moderate edema, and fibrin formation observed in lungs of shorter exposure to high oxygen atmospheres. Two notable additional changes are observed; namely, slight clumping of fibrin in a few alveoli and in a few instances perivascular edema with leukocyte infiltration.

Rabbit 3 survived 7 days' exposure to an atmosphere of 82 per cent oxygen. Grossly the lungs showed mottled areas of congestion and well marked edema. Histologically the lungs show the same changes as Rabbit 6, including the clumping of fibrin and the perivascular edema. The alveolar edema is somewhat more marked than in the preceding animal.

To summarize the examination of the lungs it can be said that exposure for 24 hours to atmospheres containing 80 per cent and

more oxygen produces little material change in the lungs. Exposure of approximately 48 hours may show nothing of moment or may lead to the development of marked congestion, edema, and early fibrinous bronchopneumonia. Exposure of approximately 3 days leads almost constantly to fibrinous bronchopneumonia which shows little change after 5 or 7 days.

The inflammatory process in the bronchi, the marked desquamation of alveolar epithelium, associated with fibrinous exudation into the alveoli, establishes the diagnosis of fibrinous bronchopneumonia. At no stage that we have observed does there appear the rich infiltration of leukocytes seen in true croupous or lobar pneumonia or in the advanced bronchopneumonia of man.

Grossly the pneumonic lungs showed an extremely variable distribution of the consolidated areas. Sometimes the process was diffuse, but even here the sharp edges of the lung frequently escaped the process; more commonly the process was irregularly distributed in small foci, 4 or 5 to the lobe. The color of the pneumonic areas was usually a deep red or bluish red, but a few areas were reddish gray. The cut surface was constantly moist and from it could be expressed frothy, salmon colored, limpid fluid. Sometimes small blocks sank in water, and others floated.

Liver.

The livers of 19 control animals were examined; 7 are absolutely normal, and 7 more show such slight changes as to be regarded as practically normal. The pathological alterations in the controls include perilobular fibrosis, coccidiosis, focal necrosis, hemosiderosis, hydropic degeneration, and in one case distinct central congestion.

Livers of Animals in High Oxygen Partial Pressures.—These organs also show the changes enumerated above in several instances, but the general picture can be presented in the statement that the principal change in the livers of animals exposed to high oxygen atmospheres is passive congestion, which may or may not be associated with hemosiderin pigmentation. This change appears in 24 hours, but is not marked until about 72 hours have elapsed, and thereafter is associated with intercellular edema, indicated by a deposit of albuminous granules between liver cells and between cell columns and sinusoidal endothelium.

Stomach and Intestine.

The gross examination of these organs showed the presence of food in all stomachs. Congestion was noted in 4 of the oxygen animals and in none of the controls. Submucous hemorrhages were found in the stomachs of 4 oxygen animals and in one control. An additional oxygen animal showed submucous hemorrhages in the upper ileum.

The gastro-intestinal tract, therefore, takes part in the general congestion found in the animals exposed to the high oxygen atmospheres.

Kidneys.

The histological examination of the kidneys of the control animals shows normal glomeruli except that in 8 of the 19 cases the loops of the glomerular tuft are unusually well filled with erythrocytes. In 4 kidneys congestion is more extensive. In 11 kidneys examined for fat, 9 show it in the loops of Henle, and in 4 of these there is additional involvement of the convoluted tubules. In 2 kidneys no fat can be demonstrated microscopically. Seven kidneys show chronic interstitial nephritis, and 4 show foci of lymphoid and plasma cells in the interstitial tissues, these changes being combined in two cases (15). Sixteen animals were examined for albuminuria before being used as controls and also after they had been in the open cage or in the chamber with practically normal oxygen content at the Nutrition Laboratory, and 5 of these on later examination showed albuminuria. Histologically, the kidneys from these 5 show chronic interstitial nephritis. Two kidneys showing chronic interstitial and one showing subacute interstitial nephritis excreted urine which was negative for albumin by the tests employed.

Kidneys of Animals in High Oxygen Partial Pressure for Approximately Twenty-Four Hours.—Six kidneys were studied in this series, and 5 show well marked general congestion. All show glomerular tufts well filled with blood. Of 3 animals examined for albuminuria, 1 was positive, the same animal being the only one of the 6 which shows a well marked chronic interstitial nephritis. Two kidneys (1 of them from the case of chronic interstitial nephritis) show albuminous degeneration to a slightly more marked degree than was found in the controls. Five were stained for fat, 4 show fat in the loops of Henle, and in 1 of these the fat appears throughout the cortex.

High Oxygen for Approximately Forty-Eight Hours.—Of the 9 kidneys in this series all but 1 show moderate or marked congestion. The subcapsular spaces of one animal show albuminous precipitate. Cloudy swelling of the epithelium of the convoluted tubules is present in all but 2 specimens. Eight specimens were examined for fat, 7 show fat in the loops of Henle, and in 2 of these the fat is found also in the convoluted tubules. The kidney of 1 rabbit shows hyalin casts in the tubules, but there is a coincident chronic interstitial nephritis. Three kidneys in this series show chronic interstitial nephritis, and 3 show subacute interstitial nephritis, the two being combined in two instances, thus making a total of 4 kidneys showing interstitial changes in this series. Albuminuria appeared in 6 of 7 examinations, in 4 instances associated with interstitial changes, in 2 cases with no marked change other than congestion.

High Oxygen for Approximately Three Days.—In this series the glomeruli are normal except for well marked congestion. The convoluted tubules show slightly more cloudy swelling than do those of the control animals. Two kidneys show fat in the epithelium of the loops of Henle. Very slight chronic interstitial nephritis is present in 3 kidneys and marked chronic interstitial nephritis in 1 other kidney. General congestion is marked in 5 kidneys and moderate in 1. Three animals had no urine in the bladder on return from the oxygen chamber. Of the other 3 only 1 showed albuminuria, and its kidney shows very slight chronic interstitial nephritis.

High Oxygen for Approximately Four Days.—Three kidneys of this series were examined histologically and all show marked filling of the tuft capillaries by blood. In 2 of these, albuminous granules are present in the subcapsular space; all 3, however, are the seat of chronic interstitial nephritis, and of these, 2 are also the subjects of subacute interstitial nephritis. Cloudy swelling is distinct in all 3, and 2 examined for fat show it in the loops of Henle. Congestion is marked in all. The one animal with urine in its bladder after return from the oxygen chamber showed no albuminuria.

The kidneys of the animals that survived 4 days', 20 hours', and 7 days' exposure show no additional changes. Congestion is marked in both and the connective tissue is normal in both. The 7 day animal shows fat in the loops of Henle; the other was not examined for fat, and neither had any urine examination.

The important change in the kidney is the appearance of congestion. Cloudy swelling appears to be more distinct in the kidneys of the experimental animals than of the controls, but this is such a fine change that its importance can easily be overestimated, particularly when the controls show minor degrees of the same process, evidently as the result of the histological technique employed. Albuminuria appeared in 8 cases, and in 6 of these the corresponding kidneys show some degree of interstitial change. Two animals showed albuminuria

after exposure of 48 hours to high oxygen. Their kidneys histologically are free from interstitial change and the greatest probability in so far as the kidney is concerned is that the albuminuria was probably due to passive congestion. Seven negative examinations following exposures of 24 hours or more would indicate that the high oxygen atmospheres, if responsible at all for albuminuria, produce it only occasionally, provided the kidney is normal at the start of the experiment, and then probably as the result of passive congestion. The 6 positive cases of interstitial nephritis are probably to be explained by the superaddition of passive congestion to a kidney already diseased, but diseased so that under ordinary conditions albuminuria did not exist.

Adrenals.

The adrenals of 16 controls were examined, and those of 14 were found to be plump organs well filled with lipoid material. Two were small with almost solid cells very poor in lipoids.

The adrenals of 16 oxygen animals show no changes sufficient to justify their grouping into sequential series. Six of the 16 show congestion in somewhat more marked degree than the controls, and 1 of these shows a small area of hemorrhage into the middle of the cortex. Fourteen show rich lipoid content and 2 do not. Two show focal necrosis, 1 in the same degree as exhibited in a control animal, and 1 shows somewhat larger and more frequent foci. This cannot be ascribed to postmortem change, as the control and the 2 oxygen animals were received alive and the organs placed in fixatives immediately after killing; furthermore, the reaction is characteristic of that taking place in living tissues.

It is therefore to be concluded that the adrenals show no change other than congestion as the result of exposure of the animals to high partial pressures of oxygen.

Spleen.

Comparatively little variation is found in the spleens of the controls except that some are more richly pigmented than others, the connective tissue content differs slightly, and the germinal centers of some spleens show more active mitosis than others.

Spleens of Animals in High Oxygen Partial Pressures.—Twenty-three spleens were examined in this series and 16 show well marked congestion. Moderate hyperplasia of endothelial cells in the pulp and sinuses is a frequent finding, but it is not more frequent than in the controls. Phagocytosis of pigment granules and of nuclear fragments is found in both controls and oxygen animals. Eleven of the 23 spleens show a few endothelial cells containing a single or only a few erythrocytes. This change is found in 2 of the control spleens; the same 2 spleens are the only controls to show congestion, and in both series this change does not occur independently of congestion. With the exception of a rabbit which was in the chamber for 2 days and 22 hours, whose spleen shows very active phagocytosis of erythrocytes, the degree of phagocytosis does not appear to be any greater in the oxygen spleens than in the controls. According to the same arguments that will be advanced in consideration of the changes in the lymph nodes and also in the general discussion, the phagocytosis of erythrocytes is regarded as part of the phenomena of congestion rather than specifically the result of the high oxygen exposure. It is therefore concluded that no specific alterations occur in the spleen as a result of the high oxygen exposure other than congestion and its incident changes.

Lymph Nodes.

Attention was not directed to the lymph nodes until after the experiments were well under way. Consequently, the number studied is smaller than is the case with other organs. Fifteen controls show large active germinal centers in all but 1 case. The sinuses show large numbers of endothelial cells, and in 10 of the nodes active mitosis is to be seen in many of these cells. In 8 of the nodes amphophilic leukocytes are to be seen in considerable numbers. Pigment is constantly present and in 4 instances large amounts are found; it occurs principally within large mononuclear and multinuclear cells of the sinuses and to a lesser degree in the large mononuclears and occasional multinuclears in the follicles. In 1 lymph node there is marked phagocytosis of erythrocytes on the part of the endothelial cells of the central sinuses. Five other lymph nodes show after careful search an occasional endothelial cell in which a few erythrocytes have been englobed.

Lymph Nodes of Animals in High Oxygen Partial Pressure for Varying Periods of Time.—Of animals exposed to high oxygen for from 24 to 72 hours 9 groups of lymph nodes were examined. Of these all show the same large proliferating germinal centers as the controls except 2 (Rabbits 24 and 49), but as these animals died in the chamber the appearance of necrosis in the germinal centers and in many of the cells in the sinuses leads one to suspect that this is largely due to postmortem change. All the nodes show considerable amounts of pigment and 5 show markedly large amounts of pigment, distributed in all cases as in the controls. Six cases show phagocytosis of erythrocytes on the part of sinus endothelium, and in 3 of these the process is marked. The most marked case of pigmentation fails to show such phagocytosis.

It would appear, from the material available, that following exposure to high oxygen atmosphere there is somewhat more marked phagocytosis of erythrocytes in the lymph nodes. Three of these cases are associated with pneumonia and 3 are not; 3 are associated with notable cardiac dilatation and 3 are not. All are associated with general congestion as observed in the kidney and spleen, and 3 are associated with similar phagocytosis in the spleen. The process appears to be more clearly associated with passive congestion in other organs than with any other demonstrable change.

Bone Marrow.

In examining histologically the bone marrow of 15 control rabbits, variations are noticeable, particularly in the proportionate amounts of fat and functionating cellular tissue. Eight of the 15 show in the fat spaces an acidophilic granular mass; in the others the fat spaces are clear. General cellular hyperplasia is found in all but 3 animals, in only 1, however, to any considerable degree. Only 1 presents a strictly normal picture, and 2 others are aplastic. One specimen shows slight general hyperplasia and also small foci of necrosis. Mitotic figures are commonly seen and fine golden brown granules of pigment, sometimes intracellular, again in extracellular position, are frequent. All but 3 specimens show a rich blood content.

The leukoblastic and erythroblastic centers, as described by Bunting (16), and the platelet formation as described by Wright (17), can be made out easily. The number of megakaryocytes appears to be extremely variable.

Specimens of marrow from 10 animals exposed to oxygen atmospheres of 80 per cent and above show no distinct departure from the pictures seen in the controls. Two are quite normal. Six show the acidophilic granular mass in the fat spaces. Hyperplasia of all the elements or of either the leukoblastic or erythroblastic centers does not exceed that of the controls. Megakaryocytes and mitotic figures are present in normal numbers. Congestion is marked in 3 specimens (Nos. 23, 24, and 49).

Erythrocyte Counts.

Erythrocyte counts were made on 12 controls before and after the experiments. The counts before experiment varied between 5,512,000 per cmm. and 8,460,000 per cmm., but were largely in the neighborhood of 6,000,000. After the experiment 7 showed increases of from 300,000 to 1,990,000 (average 1,012,000), and 4 showed decreases of 456,000 to 1,312,000 (average 768,500). One animal showed no change. The counts were made under as nearly similar conditions as possible, but these variations made it seem unlikely that the oxygen animals might show much that would be distinctive.

Five oxygen animals, the blood of which was counted before the experiment, showed variations of from 5,120,000 to 7,320,000 erythrocytes per cmm. Four animals showed an increase after exposure in the chamber of from 488,000 to 2,788,000 (average 1,313,000), and 1 showed a decrease of 1,080,000. This last animal was in the high oxygen for approximately 4 days, whereas the others varied between approximately 48 and 72 hours.

It would appear that prolonged exposure to oxygen produces no material changes in the erythrocyte count that are not observed in control animals living for similar periods under the same general conditions.

Resistance of Erythrocytes to Hypotonic Salt Solutions.

The resistance of the erythrocytes to hypotonic salt solutions was determined by the technique described in a previous communication (18). It was, of course, impossible to obtain sufficient blood for the test before the experiments, hence the control of each test was either

an animal from stock, killed for the purpose, or in several cases where 2 animals were observed simultaneously, 1 in the chamber and 1 in the room air, the latter served as a control. Of 10 control animals tested against an animal from stock, 5 showed the same corpuscular resistance, 5 showed increases in resistance corresponding to 0.050 per cent sodium chloride, and 1 showed an increase corresponding to 0.100 per cent sodium chloride.

Of 5 high oxygen animals tested against an animal from stock, 4 showed an increase of resistance equal to 0.050 per cent sodium chloride, and 1 an increase equal to 0.100 per cent sodium chloride. Of 3 animals tested with those that had been for the same time under similar conditions no change in corpuscular resistance could be demonstrated.

Reticulated Erythrocytes.

Attempts were made to determine the percentage of reticulated erythrocytes in the circulating blood, but in control animals this was found to vary between 2 and 25 per cent, a variation too wide for satisfactory work. The ground covered by this procedure is also covered by the work with resistance of the erythrocytes to hypotonic salt solutions.

Hemoglobin.

Most of the hemoglobin determinations were made with the Tallquist scale, as it was believed that to be of any importance in the rabbit, the hemoglobin difference must be large enough to show on this scale. No such differences could be demonstrated. The Dare hemoglobinometer was used with 2 controls and 2 high oxygen animals. One control showed an increase from 90 to 98 per cent, and the other a decrease from 80 to 72 per cent. Two high oxygen animals showed decreases respectively from 95 to 88 per cent and from 93 to 88 per cent, the first being in the chamber for 2 days, the second for 4 days. The hemoglobin determinations are therefore no more conclusive than the erythrocyte counts.

Leukocyte Counts.

The leukocyte count of rabbits is very variable within certain limits, and although all the counts were made at noon or early in the

afternoon (so as not to follow the late afternoon feedings), the 9 controls showed primary counts of from 5,400 to 13,200. After the experiment 4 showed increases of from 1,600 to 7,400 (average 3,950), 4 showed decreases of from 500 to 2,800 (average 2,175), and 1 remained unchanged.

Of the high oxygen animals, 1 (No. 25) showed 19,800 before and 15,100 after the experiment. No. 26, not counted before, showed 4,800 after the experiment. Of 4 others 2 showed slight increases (600 and 1,400) and 2 showed distinct increases (3,000 and 6,800) in the number of leukocytes. These may be tabulated as follows (Table I):

TABLE I.

Rabbit No.	Before experiment.	After experiment.	Increase.	Decrease.
25	19,800	15,100		4,700
26		4,800		
29	7,000	8,400	1,400	
30	8,400	11,400	3,000	
48	4,100	10,900	6,800	
56	7,400	8,000	600	

Rabbit 25 is excluded as being unsatisfactory because of the high primary count. It is safe to say, however, that Rabbit 26 shows no increase even though no primary count had been made; this animal had a distinct pneumonia. Rabbits 29 and 56 show a slight increase and were free from pneumonia. Rabbits 30 and 48 show marked increases; No. 30 had no pneumonia and No. 48 had pneumonia. It would appear that the increases are not dependent upon the presence of a pneumonia, and with the wide variations seen in control animals, variations which are not exceeded by the oxygen animals, the differences in leukocyte count are probably to be explained only as accidental variations so frequently seen in the rabbit.

Clotting Time.

Clotting time was determined by the Brodie-Russel Boggs coagulometer. Thirteen controls and 5 high oxygen animals failed to show any material differences in the clotting time as observed before and after the experimental periods.

DISCUSSION.

A discussion of the results of this work cannot be complete without calling attention to the appearance in rabbits, with a fairly high degree of frequence, of lesions not the result of experimental procedures, the so called spontaneous lesions. Without the study of numerous controls the present study might have led to false conclusions, notably in the matter of fatty degeneration of the heart. Fortunately, most of the non-experimental lesions are of subacute and chronic nature and could not reasonably be attributed to the relatively short exposure in the chamber. There is in rabbits a distinct individual variation in resistance to oxygen, as pointed out by Hill and Macleod (19), and also shown in the present series; these variations might well be accentuated by living through the diseases leading to these non-experimental lesions. There is, however, no final and conclusive evidence to support this view. The chances of error in biological experiments include this individual variation and can only be overcome by the use of a considerable number of animals.

The accuracy of study of the changes in the lungs is favored by the fact that non-experimental lesions of the lungs are rare, but on the other hand individual variations in the development of the pneumonia are fairly well marked and in the study of the lesions in this series of experiments are favored by the number of animals included. As has been shown, the lesion is a fibrinous bronchopneumonia in which edema and desquamation play a prominent part. Lorraine Smith (20) stated that fibrin plays no part in the inflammation, but our studies controvert this statement in so far as the rabbit is concerned. His statement in regard to the unimportance of leukocytes in the process is borne out in our work. The resemblance of this pneumonia to that produced by irritant gases has been noted by Bornstein and Stroink (21), and the resemblance to the earlier forms of pneumonia following nitric oxide poisoning is also to be mentioned. The absence of any well marked leukocytic infiltration in the pneumonic area and the absence of demonstrable leukocytosis in the circulating blood point toward a pneumonia of irritative rather than of bacterial origin. The lesion in oxygen poisoning develops in from two to three days and the individual variation is such that the state-

ment of Schmiedehausen and others, that the pneumonia appears in a certain number of hours, cannot be supported. The German workers particularly call attention to changes appearing in a few hours, but when compared carefully with controls the significance of these minor changes has certainly been overestimated and the percentages of oxygen used in the present series cannot be said to have produced any important changes in less than 24 hours; and exposures of approximately 24 hours have not in our experience produced any notable change other than congestion. The fact that numerous animals showed dilatation of either the right side or both sides of the heart must be considered in reference to the lung changes and the appearance of congestion of the abdominal viscera.

Studies of the influence of oxygen inhalation on circulation have not been numerous. Benedict and Higgins (22) have demonstrated a slowing of the pulse following the breathing of atmospheres containing 40, 60, and 90 per cent oxygen, and the work has been confirmed by Parkinson (23), but no studies were made directly of the pulmonary circulation. Retzlaff (24), by plethysmographic studies, has shown that oxygen produces vasoconstriction in the lungs, and he therefore argues improved pulmonary circulation. He does not show how long the vasoconstriction may persist and hence gives no conclusive evidence as to the effect of prolonged oxygen inhalation on the right ventricle.

If vasoconstriction were prolonged, it is conceivable that the right heart might fail under the demand for increased work, and in this event the pneumonia could be explained as of hypostatic origin. From the pathologist's point of view, the wide-spread involvement of the lungs argues against such an origin for the lesion. The experiments here reported give no ground for assuming that the heart dilatation precedes the pneumonia; indeed the opposite seems to be the case. The appearance of the pneumonia is more constant than of the heart dilatation, and it would appear accordingly that the heart dilatation depends upon the same circulatory difficulties which determine the same complication in human pneumonias, perhaps in these animals also influenced by the vasoconstrictor effect of the oxygen inhalation.

The general passive congestion noted in practically all the abdominal viscera is such as is seen in cases of heart failure and is to be attributed in these animals to the failure of the heart as a whole or

of its right side. Secondary changes such as cloudy swelling and even fatty degeneration can easily be accounted for by the passive congestion. The appearance of pigment in spleen and lymph nodes is so common in the controls and so inconstant in the oxygen animals that it is not to be regarded definitely as a part of the phenomena of the oxygen poisoning.

The appearance of phagocytes of erythrocytes in the lymph nodes and spleen is more frequent in the oxygen animals than in the controls. This might depend upon some disturbance of the hematopoietic balance such as is supposed to take place in splenectomized animals (25), or it may be the result of the passive congestion in these organs. The studies of the resistance of the erythrocytes to hypotonic salt solutions, the studies of the other features of the blood and of the bone marrow fail to show any distinct general disturbance of the hematopoietic system. The livers of these animals failed to show phagocytosis in the proportion of cases which would be expected were the hematopoietic system disturbed as in blood destruction in splenectomized animals. These facts and the fact that controls showing passive congestion also showed similar phagocytosis of erythrocytes in the lymph nodes and spleen leads to the conclusion that in this series of experiments this phenomenon is due to local blood destruction as the result of passive congestion and perhaps also of the small hemorrhages which frequently are seen in cases of passive congestion. It seems improbable, however, that the pigmentation of these organs, which frequently is so extensive, can be accounted for in the same way because of the short duration of the experiments.

CONCLUSIONS.

In spite of numerous abnormalities or non-experimental lesions in the rabbit certain facts can be considered as established. It has been known for many years that pneumonia is produced by the more or less prolonged inhalation of high partial pressures of oxygen. The studies herein reported show that atmospheres containing 80 to 96 per cent oxygen under normal barometric pressure produce in 24 hours, or more commonly 48 hours, congestion, edema, epithelial degeneration and desquamation, fibrin formation, and finally a pneu-

monia, probably of irritative origin and to be described as a fibrinous bronchopneumonia. The important new points are the time relations of these changes and definition of the type of the pneumonia.

Other studies have noted slight passive congestion, but it is now established that this is to be accounted for in most cases by dilatation of the right side or of both sides of the heart. This congestion affects all the abdominal viscera and is accompanied by certain secondary changes such as cloudy swelling of the parenchymatous organs and phagocytosis of erythrocytes by endothelial cells of the mesenteric lymph nodes.

Although deficiency of oxygen may affect the hematopoietic system, the animals subjected to high oxygen percentages failed to show any demonstrable pathologic changes in blood, spleen, lymph nodes, or bone marrow, except for the presence of congestion.

This study is the first of a comprehensive series projected in and under the direction of the Carnegie Nutrition Laboratory. Different animals and various methods of attack will be employed in the investigation.

BIBLIOGRAPHY.

1. Schmiedehausen, P. G., Die pathologisch-anatomischen Veränderungen der Lungen bei verändertem Sauerstoffgehalt der Atemluft, Inaugural Dissertation, Halle, 1909.
2. Bert, P., La pression barométrique, Paris, 1878.
3. Smith, J. L., The Pathological Effects Due to Increase of Oxygen Tension in the Air Breathed, *Jour. Physiol.*, 1899, xxiv, 19.
4. Heller, R., Mager, W., and von Schrötter, H., Luftdruck-Erkrankungen mit besonderer Berücksichtigung der sogenannten Caissonkrankheit, Vienna, 1900.
5. Hill, L., and Macleod, J. J. R., The Influence of Compressed Air on the Respiratory Exchange, *Jour. Physiol.*, 1903, xxix, 492.
6. Schmiedehausen, P. G., Die pathologisch-anatomischen Veränderungen der Lungen bei verändertem Sauerstoffgehalt der Atemluft, Inaugural Dissertation, Halle, 1909.
7. Schmidt, A., and David, O., Zur Chloroformsauerstoffnarkose, *München. med. Wchnschr.*, 1911, lviii, 25.
8. David, O., Versuche zur Erzeugung von Lungenhyperämie, *Ztschr. f. klin. Med.*, 1912, lxxiv, 404.
9. Bornstein, A., and Stroink, Ueber Sauerstoffvergiftung, *Deutsch. med. Wchnschr.*, 1912, xxxviii, 1495.

10. Schmidt, A., and David, O., Zur Frage der Sauerstoffvergiftung, *Deutsch. med. Wchnschr.*, 1912, xxxviii, 1697.
11. Benedict, F. G., A Respiration Apparatus for Small Animals, *Jour. Biol. Chem.*, 1915, xx, 301.
12. Brünig, A., Ueber Sauerstoffvergiftung, *Deutsch. med. Wchnschr.*, 1912, xxxviii, 1651.
13. Bornstein, A., and Stroink, Ueber Sauerstoffvergiftung, *Deutsch. med. Wchnschr.*, 1912, xxxviii, 1495.
14. Christian, H. A., Smith, R. M., and Walker, I. C., Experimental Cardiorenal Disease, *Arch. Int. Med.*, 1911, viii, 468.
15. LeCount, E. R., and Jackson, L., The Renal Changes in Rabbits Inoculated with Streptococci, *Jour. Infect. Dis.*, 1914, xv, 389.
16. Bunting, C. H., Experimental Anaemias in the Rabbit, *Jour. Exper. Med.*, 1906, viii, 625.
17. Wright, J. H., The Origin and Nature of the Blood Plates, *Boston Med. and Surg. Jour.*, 1906, cliv, 643.
18. Karsner, H. T., and Pearce, R. M., The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. IV. A Study, by the Methods of Immunology, of the Increased Resistance of the Red Blood Corpuscles after Splenectomy, *Jour. Exper. Med.*, 1912, xvi, 769.
19. Hill, L., and Macleod, J. J. R., The Influence of Compressed Air on the Respiratory Exchange, *Jour. Physiol.*, 1903, xxix, 492.
20. Smith, J. L., The Pathological Effects Due to Increase of Oxygen Tension in the Air Breathed, *Jour. Physiol.*, 1899, xxiv, 19.
21. Bornstein, A., and Stroink, Ueber Sauerstoffvergiftung, *Deutsch. med. Wchnschr.*, 1912, xxxviii, 1495.
22. Benedict, F. G., and Higgins, H. L., Effects on Men at Rest of Breathing Oxygen-Rich Mixtures, *Am. Jour. Physiol.*, 1911, xxviii, 1.
23. Parkinson, J., The Effect of Inhalation of Oxygen on the Rate of Pulse in Health, *Jour. Physiol.*, 1912, xliv, 54.
24. Retzlaff, K., Der Einfluss des Sauerstoffs auf die Blutcirculation in der Lunge, *Ztschr. f. exper. Path. u. Therap.*, 1913, xiv, 391.
25. Pearce, R. M., and Austin, J. H., The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. V. Changes in the Endothelial Cells of the Lymph Nodes and Liver in Splenectomized Animals Receiving Hemolytic Serum, *Jour. Exper. Med.*, 1912, xvi, 780. Karsner, H. T., Amiral, H. H., and Bock, A. V., A Study of the Influence of Splenectomy and of Certain Organs and Organ Extracts on the Hemopsonins of the Blood Serum, *Jour. Med. Research*, 1914, xxx, 383.

EXPLANATION OF PLATES.

Plate 20.

Fig. 1. Photomicrograph of the lung, showing marked distension of the capillaries with peripheral position of the leukocytes, but without exudation into the alveoli. This condition is not uncommonly seen in control lungs, but it constitutes also the stage of early congestion in the lungs of animals exposed for a short time to atmospheres rich in oxygen.

Plate 21.

Fig. 2. Photomicrograph of the lung, showing swelling and granular degeneration of attached alveolar epithelium, also moderate desquamation and granular precipitate within the alveoli.

Plate 22.

Fig. 3. Photomicrograph of the lung, showing advanced desquamation of alveolar epithelium with slight infiltration of lymphocytes and an occasional leukocyte.

Plate 23.

Fig. 4. Photomicrograph of the lung, showing rich fibrin formation, enclosing in the mesh desquamated epithelium, lymphocytes, and leukocytes.

THE INHIBITION OF THE TOXICITY OF URANIUM NITRATE BY SODIUM CARBONATE, AND THE PROTECTION OF THE KIDNEY ACUTELY NEPHROPATHIC FROM URANIUM FROM THE TOXIC ACTION OF AN ANESTHETIC BY SODIUM CARBONATE.*

By WILLIAM deB. MacNIDER, M.D.

(*From the Laboratory of Pharmacology of the University of North Carolina, Chapel Hill.*)

Plates 24 and 25.

(Received for publication, October 25, 1915.)

The first detailed study of the action of the salts of uranium was made by Chittenden and Hutchinson[1] and by Chittenden[2] and Lambert in two publications which appeared in 1887 and 1888. The first of these publications was concerned with the effect of various soluble salts of uranium on the amylolytic and proteolytic ferments occurring in the animal body. The authors showed that in very high dilutions these salts of uranium, and especially the nitrate, exerted an inhibitory effect upon the action of the above mentioned ferments. The second paper, although it was chiefly concerned with the effect of uranium salts upon protein metabolism, gave a brief account of the toxic effect of uranium in so far as the liver and kidney changes were concerned. The ability of uranium to induce a glycosuria was recorded, and the observation was made that the glycosuria usually did not occur until after the appearance of albumin in the urine. The appearance of glucose in the urine was attributed to a loss on the part of the liver of its glycogenolytic function.

Since these publications, uranium has been employed by a large number of investigators in the study of its effect upon metabolism,

* Aided by a grant from the fund for Scientific Research of the American Medical Association.

[1] Chittenden, R. H., and Hutchinson, M. T., The Influence of Uranium Salts on the Amylolytic Action of Saliva and the Proteolytic Action of Pepsin and Trypsin, *Tr. Conn. Acad. Arts and Sc.*, 1885–88, vii, 161.

[2] Chittenden, R. H., and Hutchinson, M. T., Some Experiments on the Physiological Action of Uranium Salts, *Tr. Conn. Acad. Arts and Sc.*, 1888–92, viii, 1.

especially protein metabolism; and on account of its toxic action on the liver and kidney it has been largely used in studying the function of these organs in various stages of poisoning. The toxic effect of uranium for the liver and for the kidney is usually ascribed to the action of the metal as such. The experiments which have been conducted in the present study tend to show that the toxicity of uranium runs parallel with its ability to lead to the formation of various acid bodies, and that if the appearance of these substances in the urine is delayed and their amount diminished there is less evidence of the toxic action of the metal.

The present paper is concerned with the toxic action of uranium nitrate on the kidney, both before and after the use of an anesthetic, and with the inhibition of this toxic action which may be induced by the use of an alkali.

In a recent paper[3] which was concerned with the study of the action of various diuretics in uranium nephritis, it was shown that two factors largely determined the efficiency of these diuretic substances. The first of these factors apparently depended upon the age of the animal, and the second on the form of anesthetic employed in conducting the experiments. Concerning the age of the animal, it was observed that before the use of an anesthetic the older animals gave greater evidence of the toxic effect of uranium than did the younger animals. This increased toxicity of uranium for the older animals was shown by the urine of these animals containing a higher percentage of both albumin and glucose, and by the earlier appearance in the urine, and by the appearance in larger amounts of both acetone and diacetic acid. The animals comprising the above series of experiments were either anesthetized by Gréhant's anesthetic, the active anesthetic ingredients of which are chloroform and alcohol, or by morphine and ether. The animals that received Grehant's anesthetic were in general less responsive to the diuretic substances than were the animals anesthetized by morphine and ether. The adult and old animals of the series which had shown an early appearance of acetone bodies in the urine, upon becoming anesthetized became acutely anuric and remained non-responsive to all diuretic substances.

The histological study of the kidneys of these acutely anuric animals has constantly shown a marked degree of swelling, vacuolation, and necrosis of the epithelium of the convoluted tubules. There was no evidence of any structural

[3] MacNider, W. deB., A Study of the Action of Various Diuretics in Uranium Nephritis, with Special Reference to the Part Played by the Anesthetic in Determining the Efficiency of the Diuretic, *Jour. Pharmacol. and Exper. Therap.*, 1912–13, iv, 491.

change of a degenerative character in the blood vessels of the kidney or in the glomeruli.

Since the publication of this paper other experiments which have been concerned with the difference in the response of animals of different ages to a constant quantity of uranium per kilo, have shown that even though the output of albumin in the urine by these nephropathic animals is of low percentage, if the output of acetone bodies was marked, the animals after being anesthetized either became non-responsive to diuretics or became acutely anuric. The kidneys of these animals have shown changes in the epithelium identical in character to the changes already described.

From the foregoing account it will be seen that there is an apparent connection between the formation of acetone and diacetic acid on the part of an animal and the toxic effect of uranium, and that the animals that show an early appearance of these bodies in the urine are more susceptible to the toxic effect of an anesthetic than the animals that either fail to show the presence of acetone bodies or show these substances in small amounts and late in the uranium intoxication.

It has been shown by Fischer[4] that an accumulation of organic acids, such for instance as lactic acid, is capable of inducing extensive tissue changes; and, also, that the use of an alkali in the form of a carbonate is capable of modifying these changes. With this observation of Fischer's in mind, and with the apparent connection between the toxicity of uranium and the toxicity of an anesthetic, with the appearance in the urine of acetone and diacetic acid, these experiments were planned in order to ascertain if the use of an alkali would in any measure inhibit the toxicity of uranium and the toxic effect of an anesthetic.

Since the commencement of these experiments the work of Graham[5] has appeared, in which he was able to show that all the characteristic features of late chloroform poisoning could be produced by the administration of hydrochloric acid, and that the use of a sodium carbonate solution would greatly inhibit the production of the characteristic lesions which develop in the liver. In his work, Graham has further shown that the ability of various narcotic substances to induce liver necrosis ran parallel with the amount of hydrochloric acid which these substances could theoretically yield in their decomposition outside of the body.

---

[4] Fischer, M. H., Oedema, a Study of the Physiology and the Pathology of Water Absorption by the Living Organism, New York, 1910.

[5] Graham, E. A., Late Poisoning with Chloroform and Other Alkyl Halides in Relationship to the Halogen Acids Formed by Their Chemical Dissociation, *Jour. Exper. Med.*, 1915, xxii, 48.

This paper strengthens the views held by Fischer concerning the important part played by various acid bodies in a variety of pathological conditions and of the beneficial effect in some of these conditions which may be obtained by the use of an alkali. It is hoped that the present paper will in some measure serve as additional evidence in this connection.

EXPERIMENTS.

The observations which form the ground for the conclusions obtained in this paper are based upon the study of the toxic effect of uranium nitrate in the dose of 5 mg. per kilo in twenty-four dogs. In previous papers[3,6] it has been shown that the toxicity of uranium is largely dependent upon the age of the animal. As a result of this observation, in conducting these experiments due consideration has been given to this factor. The two animals which have been employed in any one experiment, in which one animal served as a control for the animal receiving the alkaline solution, were either from the same litter, and therefore of the same age, or it was ascertained by careful inquiry that the animals were within a few months of the same age.

The animals were kept in metabolism cages and fed on a diet of bread and a small amount of cooked meat. They were given daily 500 cc. of water by stomach tube. The importance of diet in determining the toxicity of uranium nitrate and other poisons has been shown by Opie.[7] Animals have been shown to be more susceptible to these poisons after a diet rich in meat than following a diet rich in carbohydrate. For this reason the animals which we have employed have received very largely a carbohydrate diet. The withdrawal of the carbohydrates with an increase of the meat of the diet has resulted in an earlier appearance of diacetic acid and acetone in the urine and an increased susceptibility of the animals to anes-

[6] MacNider, W. deB., On the Difference in the Response of Animals of Different Ages to a Constant Quantity of Uranium Nitrate, *Proc. Soc. Exper. Biol. and Med.*, 1913–14, xi, 159.

[7] Opie, E. L., and Alford, L. B., The Influence of Diet upon Necrosis Caused by Hepatic and Renal Poisons. I. Diet and the Hepatic Lesions of Chloroform, Phosphorus, or Alcohol, *Jour. Exper. Med.*, 1915, xxi, 21.

thetics. After a period of observation lasting over two days, during which time the study of the urine had excluded a naturally acquired nephropathy, the animals were given subcutaneously on two successive days 5 mg. of uranium nitrate per kilo.

On each day immediately following the uranium injections, one of the animals that served as a control was given intravenously by the large saphenous vein 25 cc. per kilo of a 0.9 per cent sodium chloride solution, while the other animal was given in the same manner 25 cc. per kilo of a 3 per cent solution of sodium carbonate in 0.9 per cent sodium chloride. The animals were finally given 500 cc. of water by stomach tube and returned to their respective cages. At the end of forty-eight hours the animals were anesthetized by Gréhant's[8] anesthetic in 60 per cent strength. This anesthetic has been chosen in preference to either chloroform or ether as it is given in a definite amount per kilo by stomach and not by inhalation, and for the reason that it induces an anesthesia which is uniform in its depth and duration. This factor is, of course, of much importance in experiments of the character contained in this paper in which the toxicity of the anesthetic has to be considered.

Half an hour after giving the animals morphine, this being the initial step in inducing an anesthesia by Gréhant's method, and just prior to the use of the chloroform-alcohol mixture, the abdominal wall was anesthetized with a 2 per cent solution of cocaine, the bladder exposed, and all the urine expelled. The abdominal incision was then closed. The control animal of the experiment was then given intravenously the sodium chloride solution, while the other animal received an equivalent amount per kilo of a 3 per cent sodium carbonate solution. Immediately following these injections both animals were given, by stomach, Gréhant's anesthetic. The animals were completely anesthetized within twenty to thirty-five minutes. Usually by the end of the second hour the corneal reflex had returned. None of the animals showed any voluntary movements. The anesthesia was allowed to continue in all the animals for two hours and forty-five minutes. At the end of this time the abdominal incision

[8] The animal is given 0.25 cc. per kilo of a 4 per cent solution of morphine. This is followed in half an hour by 10 cc. per kilo of the following mixture: chloroform, 50 cc.; alcohol and water, each 500 cc.

was reopened and the urine that had been formed during the anesthesia was expelled from the bladder and measured. The kidneys and liver were then removed and the animals killed.

The Inhibition of the Toxicity of Uranium Nitrate by Sodium Carbonate.

The inhibition of the toxic effect of uranium by sodium carbonate is shown in the appended tables. A detailed discussion of this effect as evidenced by the various experiments will therefore not be given, but frequent reference to Tables I and II will be made. The tables show, in the first place, that the age of the animal influences the toxic effect of a constant quantity of uranium. If the carbonate animals be excluded from the consideration and only the response of the control animals to the uranium be taken into account, the effect of this factor—the age of the animal—is clearly seen. Thus in Experiment 3, Table I, a control animal four and a half months old, during the first twenty-four hour period following the first uranium injection, the urine contained only 0.5 gm. of albumin per liter, no glucose, and no acetone. At the end of the second twenty-four hour period, following the second injection of uranium, the urine contained only 1 gm. of albumin, 0.85 per cent glucose, and a trace of acetone. However, in Experiment 15, Table II, also a control animal, but an animal between four and five years old, the urine of the first twenty-four hour period contained 1.25 gm. of albumin, 1.67 per cent of glucose, and gave a pronounced test for acetone. At the end of the second twenty-four hour period the albumin had reached 4.75 gm. per liter, the percentage of glucose had increased to 2.06, and the urine contained not only acetone in large amount but also diacetic acid. The inhibition of the toxic effect of uranium by sodium carbonate in animals of the same age is shown in the accompanying tables by comparing the difference in the output of albumin, glucose, and acetone by the control animals with the carbonate animals.

It will be seen by referring to the tables of experiments that the animals that receive the carbonate protection show uniformly in the urine of both the first and second twenty-four hour periods a smaller

output of albumin and a lower percentage of glucose than do the animals of the same age which have not received the carbonate but which have received an equivalent volume per kilo of sodium chloride solution. It will also be seen that in the carbonate animals, with one exception, acetone was never present in the urine of the first twenty-four hour period, whereas in the control animals acetone was present in the urine of the first twenty-four hours in all animals over one year of age. In the above mentioned exception, the amount of acetone in the urine was exceedingly small. It was only detected by finding in the distillate used for the test a few characteristic iodoform crystals.

A study of the experiments shows further that with the appearance of acetone and diacetic acid in the urine, whether the animal be a control animal or an animal protected by the carbonate, there develops with the appearance of these bodies evidence of an increase in the intoxication which is shown by a rise in the percentage of glucose in the urine and in so far as the kidney is concerned in the intoxication, by an increase in the output of albumin.

The control animals have shown uniformly an earlier albuminuria than have the carbonate animals. In the urine of many of the control animals albumin has been present within eight to twelve hours after the first injection of uranium. This is true for all the control animals over one year of age. It will be noted that it is with the animals of this age that the acetone bodies first make their appearance in the urine of the first twenty-four hour period.

In the carbonate animals, however, the appearance of albumin n the urine has been delayed until the end of the first period or into the second period of twenty-four hours. With the carbonate animals it is not until this second twenty-four hour period, with the one exception which has been given, that the acetone bodies make their appearance in the urine.

From the foregoing survey of the experimental data it would appear that the toxicity of uranium is associated with the ability of the metal to lead to the production of acetone bodies, and that when sodium carbonate is employed and the formation of these bodies is delayed and their amount lessened there is less evidence of the toxic effect of the metal. The use of the carbonate in an old animal places

such an animal, in so far as its response to uranium is concerned, and in so far as the toxic effect of an anesthetic is concerned, in the relative position of a young animal in which both uranium and an anesthetic show less evidence of their toxic effect.

The following experiments are selected to illustrate the different points which have been brought out in the general review of the experiments which has just been made. Two sets of experiments will be employed: one set from the younger animals and one set from the older animals of the series.

Experiments 5 and 6, Table I, were carried out in animals between ten and twelve months old. The urine of the control animal, Experiment 5, during the first twenty-four hour period showed only a trace of albumin and glucose and no acetone. During the second period of twenty-four hours the animal developed an acetonuria, albumin was present in the amount of 1.1 gm. per liter, and glucose was present in 1.43 per cent. In Experiment 6, the animal was given the injections of sodium carbonate. The urine of the first twenty-four hour period showed a trace of albumin, no glucose, and no acetone. The urine of the second period showed only a trace of acetone, only 0.7 gm. of albumin per liter, and glucose in a lower percentage than was found in the urine of the control animal. The output of glucose at the end of the second day was 0.142 per cent. Experiments 15 and 16, Table II, were carried out in animals between four and five years of age. The urine of the control animal, Experiment 15, showed within the first twenty-four hour period acetone, albumin 1.25 gm. per liter, and 1.67 per cent of glucose. During the second twenty-four hours the amount of acetone had greatly increased and diacetic acid was present in the urine. The albumin had increased to 4.75 gm. per liter, while glucose was present in 2.06 per cent. The urine of the carbonate animal, Experiment 16, was free from acetone for the first twenty-four hours, there was no glucose in the urine, and only a trace of albumin. During the second twenty-four hour period the urine contained a trace of acetone, the output of albumin was low, 0.85 gm., and the percentage of glucose was only 0.93 per cent.

These two sets of experiments serve to show the difference in the toxicity of uranium in the animals of different ages, the inhibition

TABLE I.

The Inhibition of the Toxicity of Uranium Nitrate by Sodium Carbonate.

No. of experiment.	Age.	Weight.	Water in 24 hrs.	Uranium, 0.9% NaCl or 3% Na_2CO_3, 25 cc. per kilo.	Urine in 1st 24 hrs.	Albumin per liter.	Glucose.	Acetone.	Uranium, 0.9% NaCl or 3% Na_2CO_3, 25 cc. per kilo.	Water in 24 hrs.	Urine in 2d 24 hrs.	Albumin per liter.	Glucose.	Acetone.	Anesthetic, 0.9 % NaCl or 3 % Na_2CO_3, 25 cc. per kilo.	Duration of anesthesia.	Urine during period of anesthesia.
		kg.	*cc.*		*cc.*	*gm.*	*per cent*			*cc.*	*cc.*	*gm.*	*per cent*			*hrs.*	*cc*
1, control.	15 wks.	4.0	250	5 mg. per kg. 0.9% NaCl	212	Trace.	None.	None.	5 mg. per kg. 0.9% NaCl	250	130.5	0.75	0.71	None.	Gréhant's 60%, 0.9% NaCl	2¾	20
2, carbonate.	15 "	4.56	250	5 mg. per kg. 3% Na_2CO_3	320	None.	"	"	5 mg. per kg. 3% Na_2CO_3	250	352	0.25	0.1	"	Gréhant's 60% 3% Na_2CO_3	2¾	29
3, control.	4½ mos.	3.71	400	5 mg. per kg. 0.9% NaCl	385	0.5	"	"	5 mg. per kg. 0.9% NaCl	400	444	1.0	0.85	Acetone present.	Gréhant's 60%, 0.9% NaCl	2¾	2.5
4, carbonate.	4½ "	4.55	400	5 mg. per kg. 3% Na_2CO_3	370	None.	"	"	5 mg. per kg. 3% Na_2CO_3	400	567	0.25	0.19	None.	Gréhant's 60%, 3% Na_2CO_3	2¾	47.5
5, control.	10–11 mos.	7.79	500	5 mg. per kg. 0.9% NaCl	795	Trace.	Trace.	"	5 mg. per kg. 0.9% NaCl	500	1,040	1.1	1.43	Acetone present.	Gréhant's 60%, 0.9% NaCl	2¾	36
6, carbonate.	10–11 "	15.98	500	5 mg. per kg. 3% Na_2CO_3	745	"	None.	"	5 mg. per kg. 3% Na_2CO_3	500	900	0.7	0.142	Trace of acetone.	Gréhant's 60%, 3% Na_2CO_3	2¾	168
7, control.	1 yr.	7.83	500	5 mg. per kg. 0.9% NaCl	1,020	0.25	0.5	Trace.	5 mg. per kg. 0.9% NaCl	500	585	2.1	1.93	Present, heavy. Diacetic acid.	Gréhant's 60%, 0.9% NaCl	2¾	0
8, carbonate.	1 "	12.91	500	5 mg. per kg. 3% Na_2CO_3	95	None.	None.	None.	5 mg. per kg. 3% Na_2CO_3	500	682	0.4	0.63	Trace of acetone.	Gréhant's 60%, 3% Na_2CO_3	2¾	101

TABLE II.

The Inhibition of the Toxicity of Uranium Nitrate by Sodium Carbonate.

No. of experiment.	Age.	Weight.	Water in 24 hrs.	Uranium, 0.9% NaCl or 3% Na_2CO_3, 25 cc. per kilo.	Urine in 1st 24 hrs.	Albumin per liter.	Glucose.	Acetone.	Uranium, 0.9% NaCl or 3% Na_2CO_3, 25 cc. per kilo.	Water in 24 hrs.	Urine in 2d 24 hrs.	Albumin per liter.	Glucose.	Acetone and diacetic acid.	Anesthetic, 0.9% NaCl or 3% Na_2CO_3, 25 cc. per kilo.	Duration of anesthesia.	Urine during period of anesthesia.
	yrs.	*kg.*	*cc.*		*cc.*	*gm.*	*per cent*			*cc.*	*cc.*	*gm.*	*per cent*			*hrs.*	*cc.*
9, control.	1½–2	8.83	500	5 mg. per kg. 0.9% NaCl	820	1.9	1.46	Present.	5 mg. per kg. 0.9% NaCl	500	492	2.1	1.48	Present, heavy. Diacetic acid.	Gréhant's 60%, 0.9% NaCl	2¾	0.6
10, carbonate.	1½–2	11.61	500	5 mg. per kg. 3% Na_2CO_3	600	0.4	0.12	Trace.	5 mg. per kg. 3% Na_2CO_3	500	485	0.6	0.5	Trace of acetone.	Gréhant's 60%, 3% Na_2CO_3	2¾	114
11, control.	1½–2	9.61	500	5 mg. per kg. 0.9% NaCl	475	1.0	0.52	Present.	5 mg. per kg. 0.9% NaC_3	500	195	1.75	1.6	Present, heavy. Diacetic acid.	Gréhant's 60%, 0.9% NaCl	2¾	9
12, carbonate.	1½–2	15	500	5 mg. per kg. 3% Na_2CO_3	754	0.5	0.43	None.	5 mg. per kg. 3% Na_2CO_3	500	480	0.65	0.66	Trace of acetone.	Gréhant's 60%, 3% Na_2CO_3	2¾	95
13, control.	4–5	16.17	500	5 mg. per kg. 0.9% NaCl	1,140	1.15	0.5	Present.	5 mg. per kg. 0.9% NaCl	500	613	3.85	1.95	Present, heavy. Diacetic acid.	Gréhant's 60%, 0.9% NaCl	2¾	13
14, carbonate.	4–5	16.83	500	5 mg. per kg. 3% Na_2CO_3	485	0.3	None.	None.	5 mg. per kg. 3% Na_2CO_3	500	618	0.4	0.37	Trace of acetone.	Gréhant's 60%, 3% Na_2CO_3	2¾	151
15, control.	4–5	11.26	500	5 mg. per kg. 0.9% NaCl	1,137	1.25	1.67	Present.	5 mg. per kg. 0.9% NaCl	500	763	4.75	2.06	Present, heavy. Diacetic acid.	Gréhant's 60%, 0.9% NaCl	2¾	10
16, carbonate.	4–5	15.15	500	5 mg. per kg. 3% Na_2CO_3	1,225	0.25	None.	None.	5 mg. per kg. 3% Na_2CO_3	500	1,079	0.85	0.93	Trace of acetone.	Gréhant's 60%, 3% Na_2CO_3	2¾	95
X, uranium dissolved in 3% Na_2CO_3.	2	13.55	500	5 mg. per kg. in 3% Na_2CO_3	775	1.3	0.94	Present.	5 mg. per kg. in 3% Na_2CO_3	500	758	3.4	2.03	Present, heavy. Diacetic acid.	Gréhant's 60%	2¾	3.5

of this toxic effect by the use of the carbonate, and the relation which exists between the appearance in the urine of acetone bodies and an increase in the toxic action of the metal.

The Protection of the Kidney Acutely Nephropathic from Uranium from the Toxic Action of an Anesthetic by Sodium Carbonate.

The effect of this anesthetic on the pathology of the kidney in acute uranium nephritis, and through this effect its ability to render the kidney non-responsive to various diuretics has been shown by Pearce, Hill, and Eisenbrey,[9] and by several publications which have originated from this laboratory.[10,11]

In these later experiments it has been shown that when dogs of different ages were rendered nephropathic by uranium nitrate in the dose of 6.7 mg. per kilo and anesthetized by Gréhant's anesthetic in 60 per cent strength, the younger animals remained diuretic and responsive to such substances as caffeine, theobromine, and various salts in different molecular concentrations, while the older animals became anuric as soon as they were anesthetized and remained anuric and non-responsive to these diuretic substances. It was further shown that the failure of these substances to be of diuretic value was not dependent upon any lack of response on the part of the vascular mechanism of the kidney and that it was not due to any histological changes in this mechanism, but that the failure of these bodies to induce diuresis was constantly associated with the development in the kidney of various degrees of degeneration of the epithelium, and especially of the epithelium of the convoluted tubules.

With these observations in mind of the definite toxic effect that Gréhant's anesthetic has for the kidney acutely nephropathic from

[9] Pearce, R. M., Hill, M. C., and Eisenbrey, A. B., Experimental Acute Nephritis: the Vascular Reactions and the Elimination of Nitrogen, *Jour. Exper. Med.*, 1910, xii, 196.

[10] MacNider, W. deB., The Effect of Different Anesthetics on the Pathology of the Kidney in Acute Uranium Nephritis, *Jour. Med. Research*, 1913, xxviii, 403.

[11] MacNider, W. deB., The Vascular Response of the Kidney in Acute Uranium Nephritis,—the Influence of the Vascular Response on Diuresis, *Jour. Pharmacol. and Exper. Therap.*, 1914–15, vi 123.

uranium, and with the observation that has been made in this paper of the ability of a carbonate to inhibit the toxic action of uranium for the kidney prior to an anesthetic, both the control and carbonate animals were anesthetized by Gréhant's mixture, and the effect of the anesthetic on the kidney was determined; first, by the comparative ability of the kidneys of the control and carbonate animals to form urine during an anesthesia of two hours and forty-five minutes, and, secondly, by the difference in the severity of the pathological changes induced in the kidneys of the respective animals by the anesthetic.

A study of the tables of experiments shows quite clearly that in so far as the total output of urine is concerned during the period of anesthesia, the formation of urine is greatly favored in the animals that have been given prior to the anesthetic a solution of sodium carbonate, while the control animals that have received an equivalent volume per kilo of sodium chloride solution either secrete a much smaller amount of urine during the same period, or they become completely anuric.

For example, in Experiments 5 and 6, Table I, the animals being between ten and twelve months old, the control animal during the period of anesthesia formed 36 cc. of urine, while the carbonate animal during the same period formed 168 cc. This difference in the output of urine in the control, as compared with the carbonate animals, is seen to a more marked degree in the older animals of the experiments and serves to confirm the observation previously made of the increased toxicity of both uranium and of an anesthetic for these animals. In Experiments 9 and 10, Table II, in animals between one and a half and two years of age, the control animal during the period of anesthesia formed only 0.6 of 1 cc. of urine, while the carbonate animal had an output of urine of 114 cc.

The pathology of the kidneys of the control as compared with the carbonate animals shows the following differences. The kidneys of the control animals have shown a pronounced fatty degeneration of the loops of Henle. The kidneys of the carbonate animals have shown in all experiments less fatty degeneration of this epithelium. The most marked difference in the pathological response of the kidneys of the control animals, as compared with the changes in the kidneys

of the carbonate animals, is seen in the difference in the degree of involvement of the epithelium of the convoluted tubules. In the control animals the epithelium of these tubules shows severe injury (Fig. 1). The cells are greatly swollen and the nuclei are faint and frequently fragmented. In other of the tubules both the nucleus and cytoplasm of the cell have become necrotic. The epithelium of the collecting and junctional tubules is strikingly spared.

In the carbonate animals the epithelium of the convoluted tubules shows but slight injury and these changes are early. The terminal effect of the anesthetic on these cells, such, for instance, as a fairly complete necrosis, has not occurred in the twelve animals of the series which have received the carbonate protection.

In general the cytoplasm and nucleus of the cells of these tubules stain well. The cytoplasm is not infrequently granular, and in a few of the tubules the cells have undergone some swelling (Fig. 2). The vascular pathology of the kidney in both the control and carbonate animals has consisted in a fairly severe engorgement of the glomerular vessels, but without the appearance of any exudate into the glomerulus and without any structural changes in the endothelium of the capillaries.

From the foregoing account the conclusion appears allowable that the kidney of an animal acutely nephropathic from uranium is protected against the toxic effect of an anesthetic by the use of sodium carbonate, and, furthermore, that this protection is dependent upon the histological preservation of the renal epithelium and especially of the epithelium of the convoluted tubules.

DISCUSSION.

No attempt will be made to explain the exact mechanism through which uranium exerts its toxicity, or to explain the differences between young tissue and old tissue which apparently give to the younger animals a relative immunity to an amount of uranium which in an older animal serves as a severe toxic agent. Even though the exact mechanism of the toxic action of uranium may not be clear, we know enough of the results of its toxic effect to warrant a brief discussion of the origin of some of the tissue changes and changes in the urine.

The earliest indication of the toxicity of uranium is shown by albumin in the urine. There also appears in the urine either prior to the albumin or within a few hours of the first trace of albumin, acetone and, later, diacetic acid. Generally within twelve hours after the development of an albuminuria the animal develops a glycosuria. So far as we have been able to learn there is no attempt to explain the nephritis from uranium except through the effect of the metal on the kidney.

The glycosuria has either been explained by assuming that the glycogenolytic function of the liver is interfered with, allowing an increase in blood sugar, which finds its escape in the urine, or that the glycosuria is purely of renal origin. That it is dependent upon an injury to the kidney, and that without any increase in the amount of blood sugar the animal becomes glycosuric, is the explanation given by Wallace[12] in his study of uranium glycosuria. Neither of these explanations serves to explain the severe general toxic effect of uranium which is seen in the rapid loss of weight by the animals, or the difference in the toxicity of uranium for animals of different age, and the early appearance in the urine of acetone and diacetic acid. It appears more likely that in its toxic action, uranium has a more general effect upon cell metabolism, resembling in some respects the action of hydrocyanic acid, and that the albuminuria, glycosuria, the formation of acetone bodies, and the marked loss in weight by the intoxicated animals is an expression of this interference in a wide variety of cells.

From experiments that are now being conducted in this laboratory and from the previous work of others, we are more inclined to this explanation of the toxic effect of uranium, than to ascribe its toxicity to the direct effect of the metal on any one or more groups of cells, as, for instance, the liver or the kidney.

It has been shown by Chittenden and Hutchinson[1] in their experiments on the action of various uranium salts on certain ferments, that the nitrate exerted in very high dilutions a marked inhibitory effect upon the action of both ptyalin and pepsin.

[12] Wallace, G. B., and Myers, H. B., Uranium Glycosuria, *Jour. Pharmacol. and Exper. Therap.*, 1913–14, v, 511.

It is possible that uranium nitrate, as used in the experiments recorded in this paper, may exert a similar inhibitory effect on the oxidative enzymes of a variety of cells, and that through this action uranium induces histological changes which are common to different cells, such as acute swelling, and causes the appearance in the urine of various abnormal bodies. It would then follow from the facts observed in the experiments, that the oxidative capacity of the young animals is greater than the oxidative capacity of the older animals; for when both types of animals have been given the same amount of uranium per kilo, there is less evidence of its inhibitory effect on processes of oxidation in the young animals than in the older animals. The younger animals show a lower percentage of glucose in the urine, a later appearance of acetone bodies in the urine, and, as a result of the milder grade of nephritis, there is a lower output of albumin.

Experiments that are now being conducted have as their object a study of the oxidative capacity of the blood and various tissue juices in normal animals of different ages and in animals of different ages nephropathic from a constant quantity of uranium per kilo. They tend to substantiate the purely hypothetical explanation which has been offered for the observation that animals of different ages vary in their susceptibility to uranium and that this toxic effect of uranium is constantly associated with the appearance in the urine of acetone and diacetic acid.

In this paper it has not been shown that these bodies acting as such are the cause of the kidney damage. Other bodies similar in character may be formed in the kidney cells which are in large measure responsible for their degeneration. It has, however, been shown that the time of the appearance of these substances and the relative amount in which they appear is an index of the severity of the uranium intoxication, and that if their appearance is delayed by the use of sodium carbonate there is less evidence of the toxic effect of uranium on the kidney both prior to an anesthetic and also after a period of anesthesia.

Before arriving at this conclusion concerning the action of the carbonate it was necessary to exclude the possible detoxicating effect which sodium carbonate might have through its action on uranium nitrate, by the formation of a less toxic salt. The fact that the

different salts of uranium vary in their ability to inhibit ferment action has been shown by Chittenden and Hutchinson.[1] They were able to show that uranium acetate caused more inhibition of the action of ptyalin than did the nitrate, and they ascribed this difference to the greater acidity of the former salt. Likewise, the oxychloride of uranium showed more inhibition of the action of ptyalin than did uranium sulphate.

In order to exclude the possibility of the toxic nitrate of uranium having been detoxicated by the use of sodium carbonate, two animals were given injections of 5 mg. per kilo of uranium nitrate in which the solvent for the uranium was a 3 per cent solution of sodium carbonate in 0.9 per cent sodium chloride. As will be seen by referring to the last experiment of Table II (Experiment X), the toxic effect of uranium is in no way diminished when employed in a 3 per cent solution of the carbonate.

SUMMARY.

1. The toxicity of uranium in animals of different ages is associated with the power of the metal to lead to the formation of organic acids, as, for instance, diacetic acid and also acetone.

2. The power of sodium carbonate to lessen the toxicity of uranium depends upon its power to delay the formation of such bodies and to cause their appearance in the urine in lessened amounts, and does not depend upon the power of the carbonate to detoxicate the metal.

3. The protection of the kidney by the carbonate, which is shown by the kidney being functionally much more active during an anesthesia than the kidney of a control animal, and by the lack of fatty degeneration, acute swelling, and necrosis of the renal epithelium which is constantly seen in the unprotected kidneys, is probably dependent upon two factors: the neutralization of organic acids formed prior to and during the anesthesia, and the neutralization of hydrochloric acid which Graham[5] has shown to be liberated by chloroform during an anesthesia induced by this substance.

EXPLANATION OF PLATES.

Plate 24.

Fig. 1. Camera lucida drawing, Leitz oc. 1, obj. 6. The figure is from the kidney of the control animal, Experiment 9. The glomerular capillaries are fairly well filled with blood. In general the epithelium of the convoluted tubules shows either a severe grade of swelling with a commencing necrosis, *a*, or the epithelium is completely necrotic, *b*. The junctional tubules are not involved. One of these tubules, shown at *c*, contains a cast. The kidney formed only 0.6 of 1 cc. of urine during the period of anesthesia.

Plate 25.

Fig. 2. Camera lucida drawing, Leitz oc. 1, obj. 6. The figure is from the kidney of the carbonate animal of Experiment 10. The glomerulus shows a fairly severe engorgement. The capillaries fill the space enclosed by the capsule. The epithelium of the convoluted tubules in general stains uniformly and none of the cells are necrotic. There is shown at *a* a shrinkage of the epithelium which is frequently seen in hyperactive kidneys. At *b* an early swelling of the epithelium is shown but without necrosis. At *c* is shown in cross-section a junctional tubule, the epithelium of which is normal. The kidney formed 114 cc. of urine during the period of anesthesia.

INTRATESTICULAR IMPLANTATION OF THE FLEXNER-JOBLING RAT CARCINOMA.

By WILLIAM H. WOGLOM, M.D.

(*From Columbia University, George Crocker Special Research Fund, New York.*)

PLATE 26.

(Received for publication, November 4, 1915.)

It is generally agreed that the propagable tumors of mice and rats will proliferate in practically all the organs[1] of the body, including the testis, though the transplantability of the Flexner-Jobling rat carcinoma into this gland has not yet been definitely proved. Thus, Flexner and Jobling[2] said that their attempts to transplant this tumor into the testis had met with but indifferent success, while Levin[3]

[1] The bibliography up to 1913 is to be found in Woglom, W. H., Studies in Cancer and Allied Subjects. The Study of Experimental Cancer, New York, 1913, i. Other references are: Kraus, R., Ranzi, E., and Ehrlich, H., *Wien. klin. Wchnschr.*, 1909, xxii, 1653 (liver, omentum, and testis). Graf, R., *Centralbl. f. allg. Path. u. path. Anat.*, 1910, xxi, 723 (various organs, including the bones). Stumpf, R., *Beitr. z. path. Anat. u. z. allg. Path.*, 1910, xlvii, 571 (kidney). Levin, I., *Jour. Exper. Med.*, 1911, xiii, 604; 1912, xvi, 155 (various organs, including the brain and the bone marrow). Brancati, R., *Tumori*, 1911, i, 189 (various organs). Ruben, L., *Arch. f. Ophth.*, 1912, lxxxi, 199. Da Fano, C., *Folia neuro-biol.*, 1912, vi, 109 (brain). Uhlenhuth, P., *Deutsch. med. Wchnschr.*, 1913, xxxix, 1859 (testis and brain). Keysser, F., *Wien. klin. Wchnschr.*, 1913, xxvi, 1664; *Ztschr. f. Chemotherap., 1te Teil, Orig.*, 1914, ii, 188 (various organs, including the eye). Citron, H., *Ztschr. f. Immunitätsforsch., Orig.*, 1912, xv, 1; *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1914, lxxii, 328 (wall of stomach). Happe, *Ber. ü. d. xxxix. Versamml. d. ophthalmol. Gesellsch.*, Heidelberg, 1913, 407 (eye). Grignolo, F., *Gior. d. r. Accad. di med. di Torino*, 1914, lxxvii, 285 (eye). Ebeling, E., *Ztschr. f. Krebsforsch.*, 1914, xiv 151 (brain). A detailed account of Keysser's work is given by Hegner, C. A., an ophthalmologist in *München. med. Wchnschr.*, 1913, lx, 2722.

[2] Flexner, S., and Jobling, J. W., *Monographs of The Rockefeller Institute for Medical Research*, 1900, No. 1, 35.

[3] Levin, I., *Jour. Exper. Med.*, 1912, xvi, 149, 155; 1912, xv, 163.

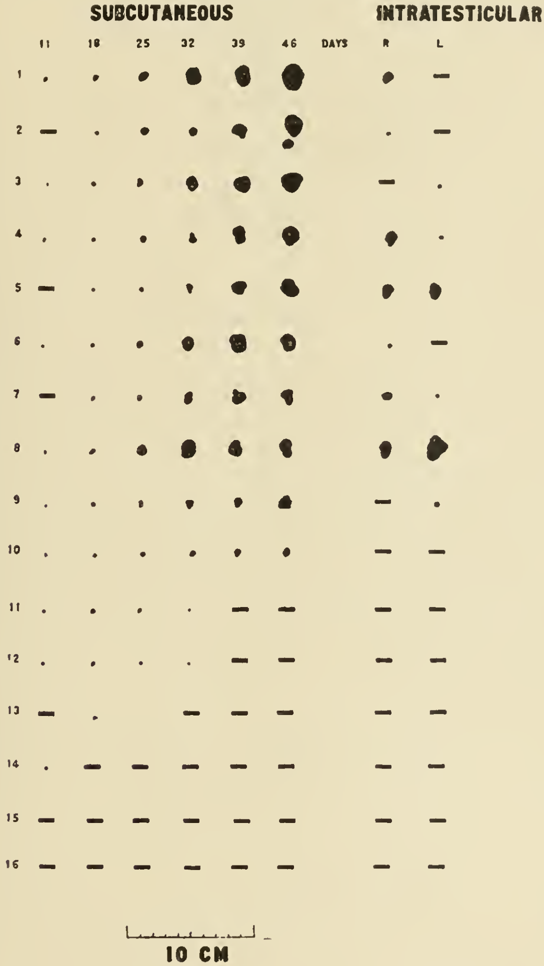
wrote that he had failed entirely to inoculate it into this organ. The failure can not be ascribed to any technical factor, because the Jensen rat sarcoma, transplanted by the same method, grew vigorously in the testis.

Since so many authors have reported the successful inoculation of other tumors at this site, it would appear from the experiments just cited that the Flexner-Jobling carcinoma is an exception to a general rule. Actually, however, it is not, since the experiments about to be described prove that this tumor grows well in the testis. A series of preliminary transplantations, which it is not necessary to reproduce here, demonstrated that the inoculation percentage of the Flexner-Jobling tumor in this gland is almost equal to the subcutaneous inoculation percentage, and that subcutaneous and intratesticular grafts in the same rat generally tend to fail or succeed together.

Although grafts do succeed, therefore, in proliferating in the testis, the resulting tumors do not attain quite the dimensions of those growing in the subcutaneous tissues. Why this should be so, it is impossible to explain. It is not true of all the parenchymatous organs, at any rate, for tumors inoculated into the kidney, for instance, attain dimensions equal to those of axillary neoplasms, as has been pointed out by Woglom.[4] The greater size of the subcutaneous growths is not apparent merely (explicable, that is, by the inclusion of skin at the weekly measurements), for in several experiments where the subcutaneous tumors were measured at autopsy, the skin was found to make but a negligible difference. The testis can not be regarded, in itself, as an unfavorable soil, for the ingrowth of blood vessels and fibroblasts during the first few days following implantation is not less marked than in the subcutaneous tissues (Fig. 1); yet it is possible, of course, that the blood supply in this gland finally becomes inadequate to satisfy the incessant requirements of a rapidly growing tumor. The presence of a growth in the axilla will not account for the smaller size of intratesticular neoplasms, since both tumors were inoculated at the same time; and ample evidence has been accumulated to show that two grafts, simultaneously implanted, do not affect one another. The question of age does not arise, for

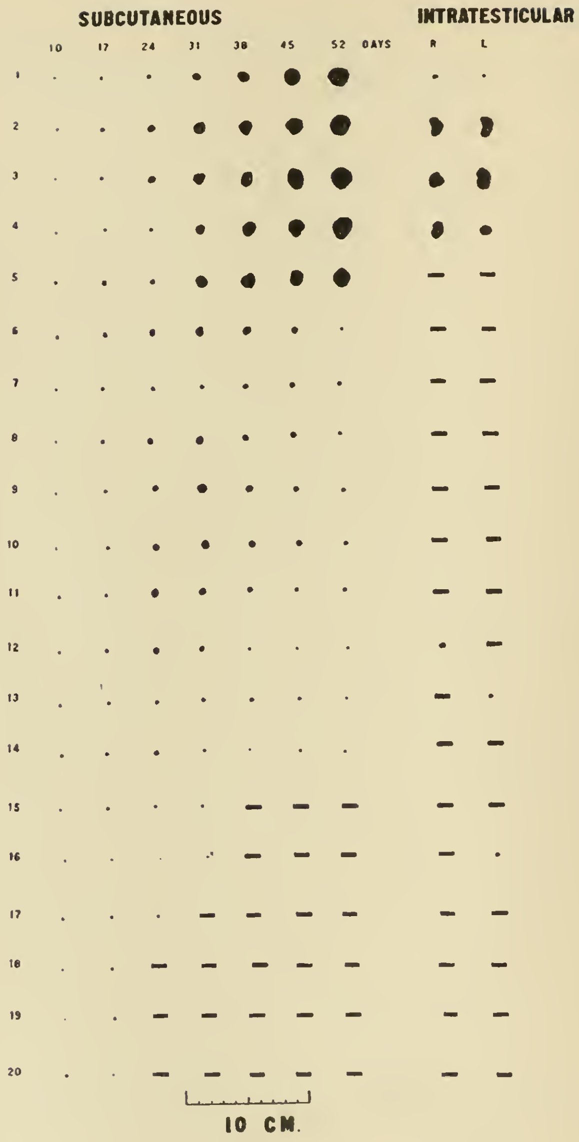
[4] Woglom, W. H., *Lancet*, 1911, ii, 92.

the animals were all young adults. The pressure of the tunica albuginea is the only remaining possibility among those that come readily

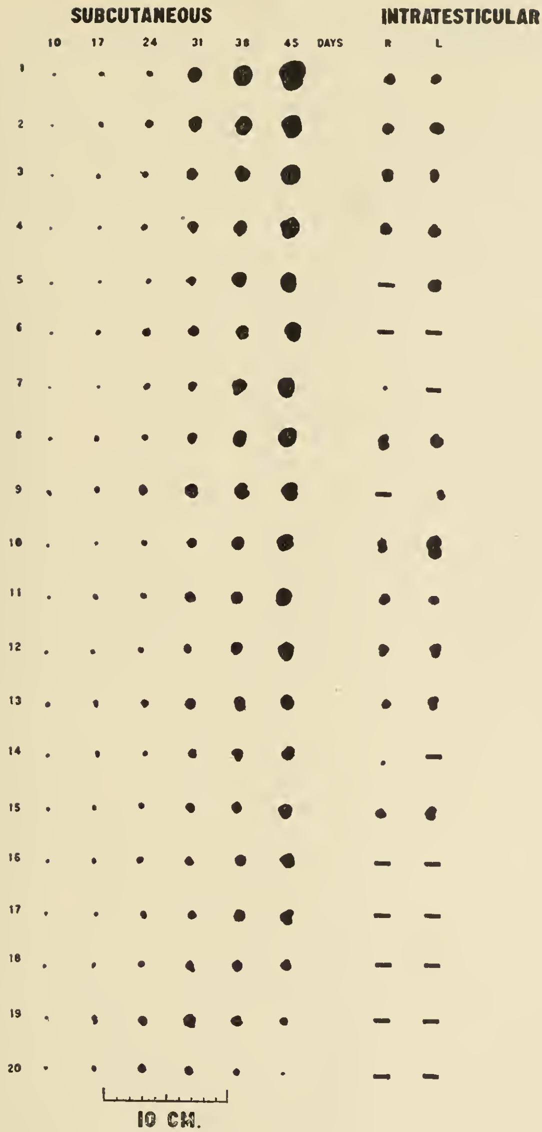


Text-Fig. 1. $\frac{FRC}{13E}$. Average weight of rats, 99 gm.

to mind; but the following experiments (Text- figs. 1 to 6) prove that this factor does not explain entirely the smaller size of intratesticular growths.

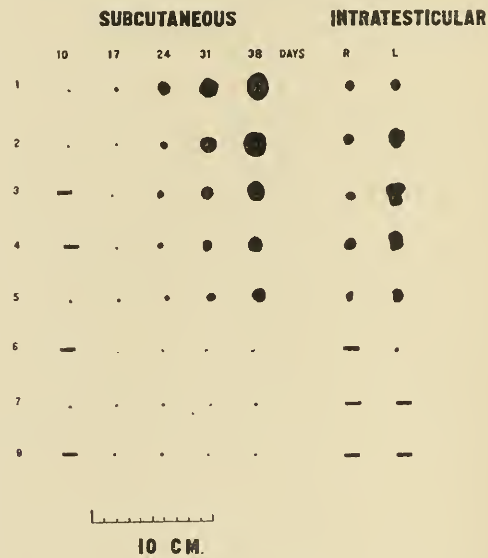


TEXT-FIG. 2. $\frac{\text{FRC}^1}{\text{14GG}}$. Average weight of rats, 79 gm.



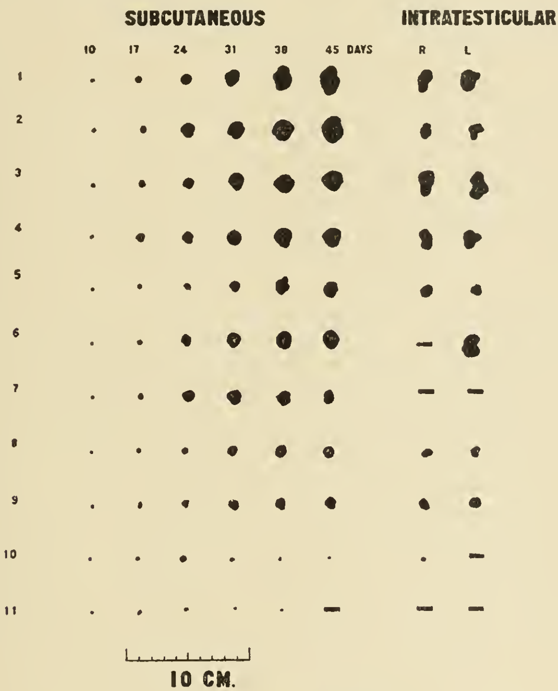
Text-Fig. 3. $\frac{\text{FRC}}{\cdot 14\text{Z}}$. Average weight of rats, 76 gm.

Six groups of young adult rats averaging 77 gm. in weight (except in experiments $\frac{\text{FRC}}{13\text{ E}}$ where the weight was 99 gm., and $\frac{\text{FRC}}{13\text{ O}}$ where the rats were not weighed), were inoculated in both testes and in the right axilla, each series at the same sitting and with an equal dose (0.01 gm. by the needle method) of the same tumor. From three to seven days afterward, the tension in the left testis was lowered by incising the tunic through an abdominal incision under ether anesthesia, while the right testis was left undisturbed as a control. The figures show the results when the rats were autopsied, from thirty-eight to fifty-two days after inoculation, the two columns to the right representing the growths in the right and left testes. The tumors in the opened (left) gland were not invariably larger than those in the unopened, as they would be were tension alone responsible for the smaller size of intratesticular growths. That the release of pressure may occasionally exert some slight favorable influence, however, is suggested by the fact that neoplasms in the opened testis had in



TEXT-FIG. 4. $\frac{\text{FRC}}{13\text{C}}$. Average weight of rats, 77 gm.

several cases grown through the incision in the tunic; furthermore, these growths were sometimes larger than those in the opposite testis. That is to say, in thirty-nine rats where grafts succeeded in both testes, a distinctly larger tumor was found in the opened gland in twelve, while in only six was the nodule on this side smaller than that in the intact organ; in twenty-one rats, the intratesticular growths were of uniform size.



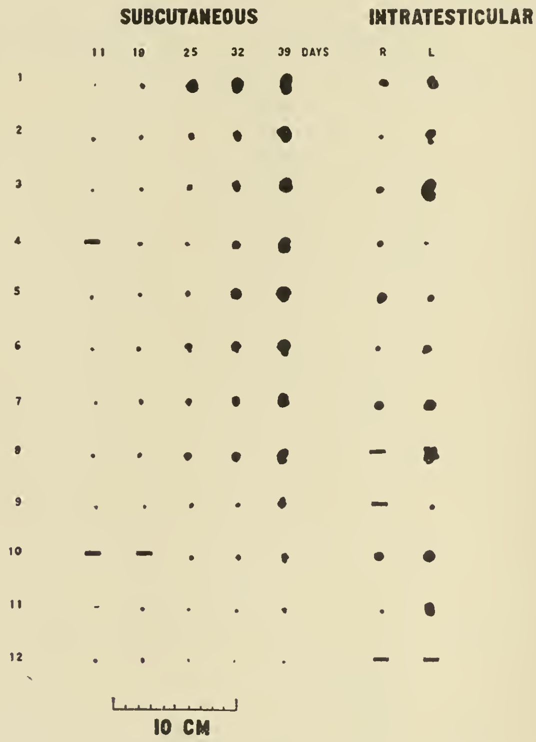
TEXT-FIG. 5. $\frac{FRC}{14V}$. Average weight of rats, 78 gm.

The absence of metastases in the abdominal cavity supports the observation of Jones and Rous,[5] that the peritoneum, in animals at least, is not susceptible to metastatic attack unless the subendothelial connective tissues have been injured.

The perfect receptivity of the various organs for inoculated tumors throws some light upon the rationale of metastasis in man. The

[5] Jones, F. S., and Rous, P., *Jour. Exper. Med.*, 1914, xx 404.

belief so long held, that the spleen, for example, actively antagonizes the growth of secondary neoplasms, has been proved untenable by the observation that tumor cells are well able to proliferate in this organ, among the lower animals at least, provided only that they succeed in reaching it. There is no reason to suppose that the human spleen enjoys a higher immunity; indeed, two authors (Kettle[6] and von



TEXT-FIG. 6. $\frac{\text{FRC}}{130}$. The rats in this experiment were not weighed.

Hansemann[7]) have but recently asserted that the resistance offered by this organ in man is not nearly so efficient as has been supposed. It is highly probable, therefore, that such exemption as it does possess

[6] Kettle, E. H., *Jour. Path. and Bacteriol.*, 1912–13, xvii, 40.
[7] von Hansemann, D., *Deutsch. med. Wchnschr.*, 1915, xli, 633.

is to be ascribed, not to the exhibition of an antagonism to the tumor cell higher than that opposed by other organs of the body, but chiefly to some mechanical factor. Such an explanation has been advanced recently by Kettle, who suggested that the contractions of the spleen force tumor emboli out of the organ again, and even prevent the growth of a large number of those that have become impacted in the capillaries.

In comparing natural metastasis with artificial metastasis, that is, with inoculation into the various organs, one distinction must not be overlooked; in the former, the tumor cells come to rest in a blood- or a lymph-channel, while in the latter process they are deposited by the needle directly in the tissues. Here they are under more favorable conditions for growth than they would be in the vessel, for a large proportion of natural emboli, unable to establish vascular connections with the vessel wall, perish in consequence, as Schmidt[8] has shown.

The work of Takahashi[9] upon artificial pulmonary metastasis, which he brought about by introducing emulsions of transplantable mouse tumors into the circulation, proves that the cells of certain of these neoplasms are unable to furnish themselves with a stroma from the vessel wall. Belonging, in general, to tumors of slow growth, they die before they have had time to effect the necessary union.

As in the experiments of Weil,[10] the tumor cells did not pass through the lungs, except in one instance. One sarcoma among the fourteen tumors employed by Takahashi (eleven carcinomata and three sarcomata) having done so, seemed to show some predilection for certain sites, resembling in this latter respect carcinomata of the prostate and the thyroid in man, which are especially apt to involve the skeletal system. Whether chemical peculiarities have any part in determining such a selective metastasis is still a disputed question; at present, however, the mechanical influences emphasized by von Recklinghausen and his pupils certainly appear to play the major rôle.

When the recent investigations of metastasis are recapitulated, it

[8] Schmidt, M. B., Die Verbreitungswege der Karzinome und die Beziehung generalisierter Sarkome zu den leukämischen Neubildungen, Jena, 1903.

[9] Takahashi, M., *Jour. Path. and Bacteriol.*, 1915, xx, 1.

[10] Weil, R., *Jour. Med. Research*, 1913, xxviii, 497.

appears that the process is governed by several factors. If a secondary tumor is to be produced, the cells of the primary neoplasm must have the power to establish vascular connections; secondly, they must remain undisturbed in the vessel long enough for this process to be completed. Thirdly, if these two conditions have been fulfilled, the organ in which they happen to lie is probably a matter of indifference in the large majority of cases.

CONCLUSIONS.

The Flexner-Jobling adenocarcinoma of the rat is easily transplantable into the testis of this animal.

The resulting growths in the intact testis do not often attain the size of subcutaneous tumors.

The smaller size of intratesticular nodules can not be explained solely by the pressure to which they are subjected during their growth; other factors, which cannot be determined, appear to be concerned.

EXPLANATION OF PLATE 26.

Fig. 1. Five day graft of the Flexner-Jobling tumor in the testis. Tumor below; testis above. Two capillaries are shown, one cut longitudinally, the other transversely. The old stroma in this field has disappeared, and its place has been taken by fibroblasts from the host. The condition is identical with that of subcutaneous grafts at the fifth day. Magnified 388 diameters.

EXPERIMENTAL SYPHILIS IN THE RABBIT PRODUCED BY THE BRAIN SUBSTANCE OF THE LIVING PARETIC.

By UDO J. WILE, M.D.

(*From the Department of Medicine and Surgery of the University of Michigan, Ann Arbor.*)

(Received for publication, November 8, 1915.)

In 1913 Forster and Tomasczewski[1] reported a method for demonstrating *Spirochæta pallida* in the living brain of patients suffering from general paresis. Their method, embodying a modification of the Neisser-Pollak trephining operation, was described by me in 1913.[2]

Forster and Tomasczewski inoculated rabbits with the cortex thus obtained, but their results were negative. In 1913 Noguchi[3] reported two successful inoculations of thirty-six rabbits inoculated with material obtained at autopsy from paretic brains. In the first case, after 97 days, typical nodules appeared in the testes and the scrotal skin. In the second, 102 days elapsed before the nodules appeared. In one case there were extremely few spirochætes, but a large number were demonstrable in the second. An attempt at inoculation into a second generation of the rabbits resulted in a very small lesion. This also appeared only after three months' time.

Berger[4] reported very few spirochætes in the lesions of three out of twenty inoculations, and he made no attempt to prolong the strain. The small number of organisms found in his case makes it probable that these were survivals.

Nichols and Hough[5] reported sixteen inoculations of brain substance from eight cases with slight success in two instances. In their cases, however, small testicular lesions resulted, with striking eye manifestations, but no spirochætes were demonstrable in the lesions, nor were they able to reinoculate other rabbits from the lesions.

All the experiments mentioned above, except those of Forster and Tomasczewski, were made from material recovered at autopsy soon after death.

---

[1] Forster, E., and Tomasczewski, E., *Deutsch. med. Wchnschr.*, 1913, xxxix, 1237.

[2] Wile, U. J., *Jour. Am. Med. Assn.*, 1913, lxi, 866.

[3] Noguchi, H., *München. med. Wchnschr.*, 1913, lx, 2484.

[4] Berger, H., *München. med. Wchnschr.*, 1913, lx, 1921.

[5] Nichols, H. J., and Hough, W. H., *Jour. Am. Med. Assn.*, 1913, lxi, 120.

During the past year I have repeated the inoculation experiments of Forster and Tomasczewski, using living material for the inoculation. It seemed to me that the lack of success of previous observers might be due to the small number of organisms present, and I therefore inoculated the brain substance of several cases into a single rabbit. Moreover, it seemed possible that the organisms from a living subject would be more likely to infect than those taken at autopsy.

For my series of experiments six cases were chosen from a large number of paretics. The diagnosis from the clinical findings and from the spinal fluid was frank general paralysis. All the cases had been under the observation of Dr. Edmund A. Christian at the Pontiac State Hospital for a long time, and in each case there were marked manifestations of advanced paresis.

The material was obtained from the brain. The site was prepared with tincture of iodine, with ethyl chloride as a local anesthetic. The skull was trephined over the frontal convolution at a point about one-half to one inch from the midline and well forward of the course of the middle meningeal artery. By means of a long thin trocar needle connected to a syringe a small cylinder of gray and white matter with some fluid from the ventricle was removed. The material was transferred to a sterile Petri dish, containing a few drops of normal salt solution and was at once examined for spirochætes under the dark-field microscope. In five of the six cases spirochætes were demonstrable; in one case they were extremely numerous, and in the remaining four it required from ten minutes' to half an hour's search to demonstrate them.

The major portion of the material thus obtained was at once injected into the testes of a large rabbit (June 11, 1915). The material from five cases was injected into the left organ and that from one case into the right. About two weeks after the inoculation, small hard nodules could be felt in both organs. Aspiration of the nodules after four weeks (July 10, 1915) showed large numbers of active, motile spirochætes. These continued to be demonstrable up to the eleventh week after inoculation, when the animal died from an accidental trypanosome infection, the nature of which was not determined. When the organisms were first demonstrable a second rabbit was inoculated (July 11, 1915) from the aspirated testicular juice of

the first animal. On August 1, 1915, twenty-one days later, both testes were found to be the sites of hard nodules, from which again, on aspiration, large numbers of spirochætes were demonstrable. These continued to be demonstrable in the second rabbit for five weeks, up to the time of the death of this animal from the same accidental infection. A third rabbit was inoculated (August 2, 1915) with the aspirated testicular juice of the second, and fifteen days after inoculation (August 17, 1915) small nodules appeared in both testes, in which again large numbers of organisms could be found on repeated examinations.

Thirty-three days after spirochætes were found in the first rabbit (August 12, 1915), the testis of one side was castrated. The organ was cut into small pieces and a mash made with normal saline solution. A portion of this material was used for cultural work and a small portion for inoculation into a fourth rabbit. At the present writing (October 28, 1915) viable organisms are still present in the cultures made from this mash. Rabbit 4 is still alive (October 28, 1915), and although no nodes are palpable in the testes, *pallidæ* in moderate numbers are still demonstrable by aspiration from both organs.

The work of Noguchi,[3] Nichols and Hough,[5] and Uhlenhuth and Mulzer,[6] seems to indicate the existence of a neurotropic strain of spirochætes. Slight differences in morphology are ascribed to this strain. According to Nichols,[7] the organism is thicker than is ordinarily found, and the curves are not so deep as in the finer variety, the organism being almost as coarse as *Spirochæta refringens*.

The spirochætes in these experiments differ in morphology from those ordinarily seen in mucous and cutaneous lesions. They were similar to those described by Nichols, being shorter and thicker. In all cases, moreover, they seemed to be less actively motile. This may have been partly due to the physical properties of the fluid in which they were studied.

Nichols and Hough reported in their inoculations the occurrence of keratitis and choroiditis from the testicular inoculations. These

[6] Uhlenhuth and Mulzer, *Centralbl. f. Bakteriol.*, *1te Abt.*, *Ref.*, 1913, lvii, Supplement, 162.

[7] Nichols, H. J., *Jour. Exper. Med.*, 1914, xix, 362.

lesions were identical with those found in acquired syphilis, but the most careful search on their part failed to demonstrate the spirochætes in their cases. None of the rabbits inoculated by me showed either the development of keratitis or other eye symptoms. The rabbit at present living, however, the fourth one inoculated, shows a patchy alopecia, particularly of the head and neck, which is strikingly like that seen in early syphilis.

Noguchi's inoculations required 97 to 102 days of incubation before the development of lesions. His inoculations in the second generation required three months before development. He concludes, therefore, that the infectiousness of general paresis for rabbits is weak and that the virulence of the spirochæte from this source to rabbits is also weak.

In my experiments the first rabbit to be inoculated was found to be infected four weeks from the time of the inoculation and undoubtedly would have proven positive before this time, as nodules were discovered two weeks earlier. The second and third generation showed an increasing virulence as exhibited by the larger numbers of organisms and by the incubation period of three weeks and fifteen days, respectively.

I desire to express my appreciation to Dr. Frederick Novy and Mr. Paul de Kruif for many laboratory courtesies extended to me, and to Dr. Edmund A. Christian for the facilities that he placed at my disposal.

CONCLUSIONS.

1. Spirochætes from the living paretic brain easily infect rabbits with experimental syphilis.
2. They constitute a virulent strain with a shorter period of incubation for the rabbit than exists with other strains.

A STUDY OF THE GASTRIC ULCERS FOLLOWING REMOVAL OF THE ADRENALS.

By FRANK C. MANN, M.D.

(*From the Mayo Clinic, Rochester, Minnesota.*)

Plates 27 and 28.

(Received for publication, November 15, 1915.)

It was noted at autopsy that animals dying after the removal of both adrenals showed acute ulceration of the gastric mucosa in a large number of cases.

Cioffi and Pende made this same observation, but Gibelli[1] was the first to give it special attention. The most extensive investigation of the subject was made by Finzi[2] in a study of the gastric mucosa of rabbits and dogs after removal of the adrenals. He found in the gastric mucosa marked circulatory changes consisting of edema, hemorrhage, necrosis, and ulceration. In the few instances in which there was a tendency to heal, the slight healing process involved only the connective tissue, never the epithelium. He found edema and hemorrhagic points in the stomach as early as one hour after extirpation of the adrenals. Dissection of the capsule of the adrenal, leaving the gland intact, did not produce ulceration. The removal of one adrenal in a rabbit produced slight and transient circulatory changes in the gastric mucosa. Animals which were given adrenalin after removal of the adrenals had normal gastric mucosa. In five cases of gastric or duodenal ulcer in man he found microscopic changes in the adrenals, consisting of thickening of the capsule, nodular hypertrophy, fatty degeneration, great congestion, and multiple hemorrhage. He believes that adrenal insufficiency may be a factor in the etiology of gastric ulcer.

Durante[3] investigated the effects of section of the splanchnic nerves on the

---

[1] Gibelli, C., La funzione delle capsule surrenali in rapporto col processo di riparazione delle fratture e coll' eziologia dell'ulcera gastrica, *Pathologica*, 1909, i, 131.

[2] Finzi, O., Über Veränderungen der Magenschleimhaut bei Tieren nach Nebennierenextirpation und über experimentell erzeugte Magengeschwüre, *Virchows Arch. f. path. Anat.*, 1913, ccxiv, 413.

[3] Durante, L., Contributo alla fisiopatologia del nervo splancnico, *Pathologica*, 1912–13, v, 631.

gastric mucosa and adrenals in dogs and rabbits. He found that section of all three splanchnics on either side produced no change except congestion in the adrenals; section of the major splanchnic affected the gastric mucosa very slightly; section of either the median or minor splanchnic on either side always produced necrosis and ulceration of the gastric mucosa. The ulcers produced by section of the nerves on the right always healed rapidly and the spleen remained normal; section of the nerves on the left produced changes in the spleen and ulcers in the stomach which tended to become chronic. He attributed the formation of ulcers after adrenalectomy to incidental injury of the minor splanchnic nerves which are buried in the posterior leaf of the adrenal.

Since this investigation was undertaken two articles bearing on the subject have appeared. Elliott[4] notes the frequent occurrence of gastric ulcer on removal of the adrenals in cats, and cites it as proof of the full digestive power of the gastric juice. He refers to the work of Finzi and concludes with the statement that gastric ulcer is not found in Addison's disease.

Friedman[5] investigated the effect on the gastric and duodenal mucosa of removal of the adrenals and one-side thyroidectomy in dogs and rabbits. He considered gastric lesions possibly dependent upon adrenal insufficiency as well as upon an excess of thyroid; duodenal lesions upon hypofunction as well as upon excess of adrenalin; coexistent gastric and duodenal lesions upon alternating conditions of hypo- and hyperfunction of the adrenals. He relies upon the pluriglandular hypothesis of an antagonistic action between the adrenals and the thyroid to explain the formation of gastric and duodenal ulcers and erosions after interference with these glands.

Dogs and cats were used in my investigation.[6] As no lesion of the gastric mucosa was found at autopsy in a series of more than 200 practically normal animals, it would seem that spontaneous ulcers are not common in these animals. Their occurrence after adrenalectomy was studied in the following series of experiments.

Experiment 1.—In four dogs the adrenals were removed at one operation and the animals kept under an anesthetic until death occurred, two or three hours

---

[4] Elliott, T. R., Some Results of Excision of the Adrenal Glands, *Jour. Physiol.*, 1915, xlix, 38.

[5] Friedman, G. A., The Influence of Removal of the Adrenals and One-Sided Thyroidectomy upon the Gastric and Duodenal Mucosa; the Experimental Production of Lesions, Erosions, and Acute Ulcers, *Jour. Med. Research*, 1915, xxxii, 287.

[6] These ulcers have also been noted in gophers dying from adrenal insufficiency, and in one case of Addison's disease.

after the removal of the last gland. In these experiments the mucosa of the stomach and duodenum was found to be normal.

Experiment 2.—In twelve dogs the adrenals were removed at two operations. When the second gland was removed the dogs were kept under ether until death occurred, which was from two to eight hours after operation. In none of these animals were gastric or duodenal lesions noted.

Experiment 3.—Forty dogs and six cats were subjected to the removal of one adrenal, usually the right, and killed at periods varying from 5 hours to 235 days after operation. In this series of animals no lesion of the gastric or duodenal mucosa was found except in one dog. This animal died from an unknown cause 4 months after removal of the right adrenal; several acute ulcers and one chronic ulcer were found in the stomach and one acute ulcer in the duodenum.

Experiment 4.—Of sixty dogs and five cats in which both adrenals were removed at the same operation or at different operations, forty animals showed lesions of the stomach, five of these ulcers of both stomach and duodenum. Of the twenty-five animals in which ulcers were not found, only four died an uncomplicated death from adrenal insufficiency; the remaining twenty-one animals were either subjected to other experiments which may have interfered with the formation of ulcers or they died before the ulcer could form.

It is seen that lesions of the gastric and duodenal mucosa did not occur in adrenalectomized animals subject to continuous etherization and were infrequent in animals subjected to the removal of only one adrenal; but lesions in the stomach and duodenum occurred in about 90 per cent of the animals dying with the characteristic symptoms of adrenal insufficiency after removal of both glands. This last fact appears significant in the study of the general causative factors of acute gastric ulcers.

It has been impossible to determine definitely the time necessary for the formation of the ulcers or how soon after complete adrenalectomy they begin to form. In only one of the animals examined within ten hours after removal of both adrenals were any changes noted in the gastric mucosa. In this instance there were several hemorrhagic areas which were possibly the beginning of ulcers. In one animal dying twenty-two hours after extirpation of both glands well formed ulcers were present. In animals examined when muscular weakness was first in evidence, beginning ulceration was noted. No changes in the gastric mucosa were found before decrease in blood pressure took place. It would appear that ulcer formation begins before the onset of the characteristic symptoms of adrenal insuffi-

ciency which progress until the death of the animal, and that only a few hours are necessary for their production.

The lesions found in the gastric mucosa after death from adrenal insufficiency consist of two main types: one is a wide-spread, superficial erosion; the other is a true, punched-out ulcer formation.

The gastric erosions practically always occurred in the fundic division, and in most cases the pyloric mucosa appeared normal. They appeared to begin in and spread along the rugæ, thus producing an irregular appearance. Only the surface of the mucosa was affected, the loss of epithelium never extending to the submucosa. The denuded surface was hemorrhagic in appearance, and the fluid in the stomach was usually blood-stained. This condition developed mainly in those animals in which there was a prolonged moribund condition following the development of muscular weakness.

The gastric ulcers were round or oval in shape and varied in size from 2 mm. to 2 cm. in diameter. They were usually multiple, but a few stomachs contained only one ulcer. Their position varied; they were found in the fundic and pyloric regions on both the greater and lesser curvatures. Usually they occurred in the prepyloric division. Beginning ulcers appeared as small hemorrhagic areas; when fully formed, however, they penetrated to the muscularis mucosa with a complete loss of epithelium. The walls were smooth, giving the characteristic punched-out appearance. A small blood vessel was usually found in the base of the ulcer. When the autopsy was performed immediately after death, in many instances the vessels were bleeding. In the pyloric region the blood gave the ulcer a black appearance, while in the fundus it often remained bright red at the site of the ulcer. In most instances the ulcers constituted the only pathologic change in the mucosa, while in other specimens the mucosa was injected throughout (Figs. 1 and 2).

The duodenal mucosa was usually congested in the adrenalectomized animals. In five experiments there were definite ulcers. These duodenal ulcers occurred just distal to the pyloric ring and appeared like cauterized areas about 1.5 cm. in diameter. They were deeper at the center than at the edges. They penetrated to the muscularis mucosa at the center. They never showed evidence of hemorrhage (Fig. 2).

Microscopically, the picture of the ulcer varies slightly; usually, however, they are cone-shaped with the base of the cone at the surface and the apex at the muscularis mucosa. The edges of the ulcer are clean, although occasionally there may be some cellular débris and blood at the base (Fig. 3). None but the earliest signs of healing have ever been observed.

The loss of tissue appears to begin at the surface. The gland cells disappear first, allowing the supporting tissue to fall together. In some cases the ulcer has not extended to the muscularis mucosa. In these ulcers the gland cells below the base may appear perfectly normal. Hemorrhage seems to be of early occurrence in the formation of the ulcer. In practically all ulcers the blood vessels in the vicinity are congested. The special stains for mucin demonstrate the fact that this substance is usually absent near the ulcers (Fig. 4). The glands in the vicinity of the ulcer may appear normal, but do not contain mucin. The zymogen content of all the cells is decreased, but no more so in the cells around the ulcer than elsewhere.

It has been suggested that the regurgitation of pancreatic secretion is the cause of gastric ulcers and that tryptic ulcer would be a more exact term than peptic ulcer. That the pancreatic secretion is not necessary for the formation of these ulcers was proved by a series of five experiments in which the pancreatic ducts were either doubly ligated and sectioned, or a pancreatic fistula was made before the removal of the last adrenal. In these animals no pancreatic secretion could reach the gastro-intestinal tract, yet many characteristic gastric ulcers were found after death.

It has been demonstrated that bile in association with a strongly acid gastric juice has an erosive action on the gastric mucosa.[7] That the bile might be of importance in the production of these ulcers seemed possible because of the fact that the fluid found in the stomach was usually bile-stained. In one animal the common bile duct was transplanted to the skin, thus making it impossible for any bile to enter the stomach. After complete recovery from the operation,

[7] Smith, G. M., An Experimental Study of the Relation of Bile to Ulceration of the Mucous Membrane of the Stomach, *Jour. Med. Research*, 1914, xxx, 147.

the adrenals were removed. Well formed ulcers were found at autopsy.

The gastric content was always acid in the adrenalectomized animals. In order to determine the part the acid played in the production of the ulcers, an attempt was made to neutralize it during the moribund period. To accomplish this sodium bicarbonate was administered about every four hours, either in solution by stomach tube or in capsules. Of course, it was impossible to be sure that the gastric contents were always kept neutralized, but certainly in most instances no great excess of acidity developed. In a series of ten experiments in which sodium bicarbonate was administered after the removal of the last adrenal, ulcers were found in one animal only. In this experiment it is possible that the bicarbonate was not given frequently enough to prevent the development of acidity. This result would tend to show, as has been demonstrated in regard to the formation of other acute ulcers, that acid is a factor in their production.[8]

In a series of ten experiments a gastro-enterostomy was performed during the interval between the removal of the adrenals. In only four of these animals were ulcers found. It is possible to explain this result as due to a reflux of the alkaline intestinal secretion and the bile.

SUMMARY.

Acute ulcers of the gastric mucosa are found in a large percentage of dogs and cats dying after adrenalectomy. These ulcers seem to develop during the moribund period. They are apparently peptic ulcers forming at the site of local hemorrhages in the gastric mucosa. They are true acute ulcers, usually penetrating to the muscularis mucosa with a total loss of epithelium. They develop in the absence of pancreatic secretion and bile. However, they appear to develop only in an acid medium.

[8] Bolton, C., Ulcer of the Stomach, London, 1913, 59.

EXPLANATION OF PLATES.

PLATE 27.

FIG. 1. Gastric mucosa of a dog, showing multiple acute ulcers.

FIG. 2. Pyloric and duodenal mucosa, showing multiple acute ulcers.

PLATE 28.

FIG. 3. The center of a gastric ulcer, showing the clean edges and base.

FIG. 4. The edge of a duodenal ulcer, showing the loss of goblet cells (the dark stained cells) in the vicinity of the ulcer.

A TRANSPLANTABLE CARCINOMA OF THE GUINEA PIG.

By F. S. JONES, V. M. D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 29 TO 32.

(Received for publication, September 15, 1915.)

The successful transplantation of rat and mouse tumors has led to many attempts to transfer the tumors of other species. The venereal lymphosarcoma of dogs, transferred by coitus, has been studied by a number of investigators, especially Sticker.[1] Von Dungern and Coca[2] described, and succeeded in transferring an epidemic tumor of the hare. They[3] were able to transfer the venereal dog tumor to foxes and the hare tumor to rabbits. The status of these growths must be considered doubtful since it has been well established that neoplasms in general are only transplantable within the species in which they occur spontaneously. Ribbert[4] has described a fibroma of the dog which he inoculated successfully into two other series of dogs.

Rous[5] succeeded in transferring a spindle-celled sarcoma of a fowl. Later he and his collaborators reported a number of other transmissible avian sarcomata. Fujinami and Inamato[6] have transplanted a myxosarcoma of the fowl. More recently Schultze[7] described a spindle-celled sarcoma of the rabbit which he had propagated through twelve tumor generations. In the laboratories of the Imperial Cancer Research Fund of London another rabbit sarcoma has been studied.

Up to the present a transplantable tumor of the guinea pig has not been reported. Few data are obtainable concerning the tumors of this species; apparently they are rare. Sternberg[8] reported a metastasizing adenocarcinoma of the mammary gland of the guinea pig. He did not attempt transplantation.

[1] Sticker, A., *Ztschr. f. Krebsforsch.*, 1904, i, 413.

[2] von Dungern and Coca, *Ztschr. f. Immunitätsforsch., Orig.*, 1909, ii, 391.

[3] von Dungern, *München. med. Wchnschr.*, 1912, lix, 238.

[4] Ribbert, H., *Centralbl. f. allg. Path. u. path. Anat.*, 1910, xxi, 625.

[5] Rous, P., *Jour. Exper. Med.*, 1910, xii, 696.

[6] Fujinami, A., and Inamato, K., *Verhandl. d. jap. path. Gesellsch.*, 1911, i, 114.

[7] Schultze, W. H., *Verhandl. d. deutsch. path. Gesellsch.*, 1913, xvi, 358.

[8] Sternberg, C., *Verhandl. d. deutsch. path. Gesellsch.*, 1913, xvi, 362.

The tumor which I have transplanted occurred in the mammary gland of an old female guinea pig which, judging from the condition of the breast, had recently suckled offspring. This animal was removed from the Institute breeding pens, with a number of others suffering with abscesses. It had a large fluctuating abscess in the submental region and a large, rather firm, sharply circumscribed tumor in the left mammary gland. This tumor lay in the substance of the gland directly beneath the skin, but was not attached to the subcutaneous tissue or musculature. It was hemispherical in shape, of a grayish white color, and measured 4.5 by 4 by 3.6 cm. The mass was enveloped in a delicate, translucent capsule. It was removed and cut across. Just beneath the capsule was a zone of grayish white, translucent tissue 0.5 to 1 cm. wide. A milky fluid exuded from its cut surface. Toward the center of the tumor this zone gradually faded off into a rather granular necrotic material. The color of this portion of the growth varied from yellowish white to ocher. The diagnosis of cancer was made from frozen sections of the outer zone of grayish white tissue. Examinations of the regional lymph glands and other organs for metastases were negative.

Bits of the peripheral layer of the tumor were inoculated by means of a trocar into the subcutaneous tissue of the groin and into the muscles of the upper leg of sixteen young guinea pigs. Most of the animals were between two and three weeks old. Animals of this age were chosen because it has been frequently shown that young individuals are especially favorable hosts for tumor grafts.

First Tumor Generation.

Oct. 30, 1914. Sixteen young guinea pigs were inoculated with two small pieces of the tumor in the subcutaneous tissue of the left groin and the muscles of the left upper leg. The pieces varied from 0.15 to 0.2 cm. in diameter.

On Jan. 15, 1915, one of the animals showed a swelling in the muscles of the leg at the site of inoculation (Fig. 1). The growth was irregularly rounded and protruded sharply from the surface of the leg. It measured 2.1 by 1.7 by 1.2 cm. The subcutaneous graft in the left groin could not be detected on palpation.

On Jan. 20, 1915, the animal was anesthetized with ether and the major portion of the tumor removed. It lay within the muscles, protruding sharply from the general contour of the leg. Externally it was covered with a thin sheet of muscle. The mass was encapsulated on its external surface with a thin, trans-

lucent layer of connective tissue. The inner surfaces were firmly fixed to the muscles. Nearly all of the tumor was removed in small pieces. These consisted of pink, friable tissue, some portions of which were hemorrhagic. A few circumscribed areas of necrosis were noted. The incision in the skin was closed with silk. Most of the tissue was used to inoculate another series of animals. The remainder was fixed in Zenker's fluid for microscopic study.

The other animals of this series were under observation for over 6 months. None of them developed tumors.

Histology of the Primary Tumor.

Sections of the original tumor show it to be a carcinoma. The cells are arranged in acini, or more or less irregular clumps, supported by a delicate connective tissue stroma. The proliferating portion is confined to a narrow layer surrounded by a thin capsule. In one section the cancer cells have penetrated this capsule and invaded the mammary gland (Fig. 5). The individual cells in the growing portion are large, with a large vesicular nucleus. They stain well. A few mitotic figures are seen. Toward the center the cells appear shrunken and the nuclei stain intensely, while still further in all cell structure is lost. The tumor is well vascularized, but not hemorrhagic.

A few eosinophils are found along the borders of the tumor. In several places its cells have invaded the fatty tissue of the gland (Fig. 5). The interacinar connective tissue is increased throughout the section. Many of the neighboring acini and ducts show pressure changes. They are flattened and the epithelium is atrophied. The wall of one of the larger ducts adjacent to the tumor has become infiltrated with tumor cells and the growing tissue extends as a considerable mass into the duct lumen. There is a marked collateral hypertrophy of the lining epithelium and the interacinar mass is partly covered by a double layer of non-malignant columnar cells (Fig. 7).

Of great interest are certain changes in the breast that cannot be attributed directly to the tumor. One finds the atrophy, overgrowth of connective tissue, and chronic inflammatory changes seen in the breasts of old animals of other species. Some of the acini at considerable distance from the tumor show proliferative changes. Their epithelium has proliferated until they are nearly occluded with a densely packed mass of cells (Fig. 6). Apparently the cells in lesions

of this type show no tendency to break through the acinar walls. A few acini reveal still more advanced changes and have almost lost their character. The remains of such an acinus consist of an irregular clump of cells with an ill defined basement membrane. As a whole, the changes are similar to the so called precancerous lesions noted by Haaland[9] in the breasts of old female cancerous and non-cancerous mice. McCarty[10] has also described the same conditions of the mammary glands of women suffering from carcinoma of the breast.

The Tumor of the First Transplantation.

It has been stated in the protocol that a tumor developed slowly in one animal inoculated with small pieces of the spontaneous tumor (Fig. 1). The greater portion was removed, but it began to recur 3 weeks after operation.

Mar. 3, 1915. The operated tumor remained quiescent for about 3 weeks and then began to grow rapidly. On this date it measured 2.4 by 1.5 by 1.5 cm. The enlarged inguinal lymph gland on the same side was felt as an oval disc measuring 1.9 by 1.4 by 0.6 cm. (Fig. 2). Growth of the subcutaneous graft had not taken place.

Mar. 8, 1915. The animal was found dead. The leg tumor measured 2 by 1.6 by 1.2 cm. It was an irregular flattened sphere with a smooth projecting surface. The deeper portions had invaded the muscle. On section the growth was divided into numerous lobules by strands of connective tissue. Considerable brownish yellow, necrotic material was noted. The peripheral portions of the tumor were composed of friable, translucent, yellowish white tissue which exuded a milky fluid.

The disc-shaped mass in the inguinal region proved to be a metastasis in which the lymph gland had been replaced almost entirely with tumor tissue. It measured 1.2 by 1.1 by 0.6 cm. and it was made up of the same type of tissue as the leg tumor. About 1.5 cm. above the metastasis there were two tiny, discrete, hemispheral, grayish white nodules on the external surface of the abdominal muscle. These were doubtless a part of the tissue supposed to have been inoculated subcutaneously.

On opening the abdominal cavity a large, irregular, ovoid tumor was found. It measured 6.6 by 5.0 by 3.8 cm. and lay on the right side between the liver and

[9] Haaland, M., *Fourth Scientific Report of the Imperial Cancer Research Fund*, 1911, 1.

[10] McCarty, W. C., *Surg., Gynec. and Obst.*, 1913, xvii, 441.

cecum (Fig. 4). Broad bands of connective tissue connected it with the neighboring viscera and peritoneum. Its surface was smooth and glistening. The mass was divided by fissures into four principal lobules which in turn were separated into a large number of small lobulations. The color varied. The sounder portions were grayish white and translucent. Considerable hemorrhage had occurred beneath the capsule, which resulted in a gray and red mottling. The capsule was thin and well vascularized.

On section the surface lobulations were found to extend throughout. The four principal ones were separated by broad bands of connective tissue. At both ends and along the superior border was a layer of translucent, grayish white, nearly homogeneous tissue. The balance of the tumor was practically comprised of pinkish or reddish gray necrotic material.

Other tumors were not found in the abdominal cavity. Gross or macroscopic metastases were not observed in the other organs or glands.

Doubtless the large abdominal tumor was responsible for the death of this animal. From the location and general character of the tumor it seemed evident that it resulted from direct transplantation. Probably the cannula, with which the subcutaneous inoculation had been made, pierced the abdominal wall, and a small portion of the tumor lodged in the mesentery. The inguinal tumor was unquestionably a metastasis from the leg mass to the inguinal lymph gland.

Second Tumor Generation.

Series A.—Jan. 20, 1915. Thirty young guinea pigs were inoculated in the leg muscles and in the subcutaneous tissue of the groin with small pieces of tumor from No. 31.

Feb. 26, 1915. One animal, No. 26, had developed a tiny shot-like nodule in the leg and groin.

Mar. 8, 1915. Animal 26 was found dead. In the muscles of the leg at the site of inoculation were three round, raised, grayish red nodules varying in size from 0.2 to 0.4 cm. In the subcutaneous tissue of the groin 6 small glistening gray nodules were found embedded in the fascia. Microscopically these consisted of living tumor tissue, doubtless that which had been implanted. Metastasis to the other organs had not taken place. Bronchopneumonia caused the animal's death.

Mar. 8, 1915. Two other guinea pigs, Nos. 9 and 38, had developed small tumors in the leg and groin. The greater portion of the leg graft of No. 9 was removed and inoculated into the leg muscles of twenty young animals (3rd Tumor Generation, Series A).

May 1, 1915. The operated tumor recurred very slowly at first, but later grew rapidly. On this date it measured 2 cm. The animal was again anesthetized and a considerable portion of the leg tumor removed; bits of it were injected into twenty-five others (3rd Tumor Generation, Series B).

May 11, 1915. The leg tumor of No. 38 had grown slowly. At this time it measured 1.8 cm. in diameter. The major portion of it was removed, chopped fine, and drawn into a syringe. Five young guinea pigs were inoculated in the leg muscles with small amounts of the hash.

May 15, 1915. The tumor of No. 9 enlarged rapidly after the last operation, but soon began to soften and become a huge, almost spherical swelling. It involved the whole upper leg and hip and extended to the mammary region (Fig. 3). It measured 4.7 by 4.2 by 4.2 cm. In the leg of Guinea Pig 38 a large diffuse wedge-shaped nodule had appeared.

June 18, 1915. Animal 9 was found dead. The leg tumor had begun to ulcerate several days before. At autopsy it was found to be largely necrotic. The inguinal lymph glands draining the tumor were enlarged. Gross tumors were not found in other organs. Microscopic examination of the enlarged inguinal lymph glands revealed a metastatic tumor in one of them. Metastatic tumor cells were observed in sections of the kidney. They were scattered in irregular aggregates in the external portion of the medulla. The process seemed to have begun in the interlobular capillaries with consequent invasion and obliteration of the tubules. Metastases were not found in the lungs or other organs.

Series B.—Mar. 8, 1915. Twenty young guinea pigs were inoculated by means of a cannula in the leg muscles and in the peritoneal cavity with bits of the leg and abdominal tumors of No. 31, obtained some hours after death. The animals were under observation over 4 months, but none of them developed a tumor.

Third Tumor Generation.

Series A.—Mar. 30, 1915. Twenty young guinea pigs were inoculated with cannulas in the left leg with bits of tumor from No. 9.

Apr. 16, 1915. No. 103 had a small tumor at the point of inoculation.

May 11, 1915. Three animals, Nos. 103, 77, and 67, had developed tumors of a rather uniform size in the inoculated leg muscles. The largest measured 2.3 cm. and the smallest 1.8 cm. On operation most of the tumor of No. 103 was necrotic. Five young guinea pigs were inoculated in the leg with the better portions of the growth (4th Tumor Generation, Series A). Twenty others were injected in the leg with small pieces of tumor incised from the leg of No. 77 (4th Tumor Generation, Series B).

June 16, 1915. The tumor of No. 67 was incised and several small portions of it were inoculated into the upper leg of 15 young guinea pigs (4th Tumor Generation, Series C).

The Transplanted Tumor.

There has been a gradual increase in the number of takes. In the later generations the tumors have appeared earlier and have grown more rapidly and reached a larger size.

Microscopically the transplanted tumor consists of a zone of variable width of rapidly proliferating epithelial cells surrounded by a thin capsule. The cells may be grouped in acini, but they usually lie in small irregular clumps. Large numbers of them may be undergoing mitotic division (Fig. 9). Degenerative changes similar to those observed in the spontaneous tumor are frequent. At the growing edge small blood vessels are numerous. Occasionally they have the form of thin-walled sinuses (Fig. 8). One is struck by the slight reaction about the tumors; practically no round celled infiltration is observed about their borders. The connective tissue capsule is well defined but thin.

SUMMARY.

An adenocarcinoma of the mammary gland of an old guinea pig has been successfully transplanted through eight successive series of animals. It now appears much earlier and grows more rapidly. The number of takes also has increased. In two instances metastasis to the regional lymph glands (inguinal) has been observed. Once microscopic metastases were found in the kidney. The so called precancerous changes observed in the breasts of women and mice suffering from mammary carcinoma were found in the mammary gland of the spontaneous tumor animal. It is hoped that the tumor may soon be utilized for experimental purposes.

EXPLANATION OF PLATES.

Plate 29.

Fig. 1. The tumor in the leg of Guinea Pig 31 of the 1st Tumor Generation, 81 days after inoculation.

Fig. 2. Recurrence in the leg of the same animal, at autopsy 47 days after partial removal. Note the metastasis to the inguinal lymph gland.

Fig. 3. Large cystic tumor of the leg of Animal 9 of the 2nd Tumor Generation, 4 months after inoculation. On two different occasions large portions of the growth had been removed.

Plate 30.

Fig. 4. The abdominal tumor found at autopsy of No. 31 of the 1st Tumor Generation. It has been reflected to show the bands of attachment.

PLATE 31.

FIG. 5. Cells of the spontaneous mammary tumor invading the fatty tissue of the gland.

FIG. 6. Epithelial inclusions in the lumen of the mammary acini of the original animal.

FIG. 7. Growth of the cells of the spontaneous tumor through the wall and into the lumen of one of the larger milk ducts. The growth of the cancerous epithelium has been accompanied by a collateral growth of the lining columnar cells. Note the pressure changes of the smaller ducts.

PLATE 32.

FIG. 8. The characteristic border of growing neoplastic cells with many blood sinuses in a section of transplanted tumor.

FIG. 9. The transplanted tumor in a later generation showing the usual arrangement of cells. Many of them are undergoing mitosis.

THE PRESERVATION OF LIVING RED BLOOD CELLS IN VITRO.

I. METHODS OF PRESERVATION.

BY PEYTON ROUS, M.D., AND J. R. TURNER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 15, 1915.)

There is practically no mention in the literature of attempts to keep red blood cells alive for a long time *in vitro*. Yet methods for their preservation might have much practical importance, and certainly would possess theoretical interest. Kept cells could be utilized for serum reactions, or for culture media, or even under certain circumstances for transfusion. This last possibility is the more worth considering because of the recent experiments of Abel and his coworkers[1] on plasmaphaeresis. They have demonstrated that the body tolerates well the repeated withdrawal of large amounts of plasma, rapidly placing new fluid in circulation; from which it follows that only the formed elements of blood need be supplied to a healthy animal depleted by hemorrhage.

The essential peculiarities of the red blood cells must be taken into account in any attempt to preserve them. They have little of the ability to adjust themselves to changes in external conditions which is possessed by many somatic cells[2] in common with the unicellular organisms. As bits of protoplasm without a nucleus, multiplication is impossible to them, and their existence is necessarily limited, whether they are in the circulation or *in vitro*. If they are to be kept alive outside the body, it must be in what one might term a state of suspended

[1] Abel, J. J., Rowntree, L. G., and Turner, B. B., *Jour. Pharmacol. and Exper. Therap.*, 1914, v, 625.

[2] Striking instances of this ability are afforded by the *in vitro* growth of tissue cells in plasma diluted with distilled water, in plasma from other animal species, in synthetic media, etc.

animation. Experience has taught that they are best kept in the cold. But even then whether the medium be serum or physiological salt solution, they break down within a few days,—much sooner than they are supposed to do in the circulation. Evidently there is room here for improvement in method, or else current estimates of the length of life of the red cell are faulty.

Injury during Washing.

For our experiments we have used cells of the rabbit, dog, sheep, and man. It seemed best in the initial tests to free the cells of plasma and suspend them in solutions of known constitution. Meltzer[3] has shown that red cells shaken in plasma to the slight extent necessary for defibrination break down much sooner on keeping than when the blood is allowed to clot undisturbed. We have asked ourselves whether injury during washing might not be responsible for the brief survival of washed cells. That in the case of dog, rabbit, and sheep cells, especially the first mentioned, some injury may occur is evident from the hemolysis frequently observed during washing even when the cells are handled most carefully.

Experiment 1.[4] *Cells Washed and Kept in Ringer's Solution Break Down Sooner than Those Left Undisturbed in Citrated Blood.*—Dog blood was taken in Ringer's solution containing 1 per cent of sodium citrate in the proportion of 1 part of blood to 4 of the solution. Some of the mixture was at once tubed and set aside. The cells of the remainder were washed twice with ordinary Ringer's solution and distributed in Ringer's plus citrate and in Ringer's solution, respectively, to the same amount as in the original blood-citrate mixture. The tip of the pipette with which the washing was carried out was kept beneath the surface of the fluid, and in other ways the handling was careful. There was no immediate hemolysis. Tubing was done as in the case of the unwashed blood. The preparations were kept in the ice box.

Two days later hemolysis was well marked in the preparations of washed blood. The cells of the original citrated blood were still intact.

Experiment 2. Cells Washed and Kept in a Plasma-Locke's-Citrate Mixture Hemolyze Sooner than Those Left Unwashed in the Same Mixture.—Rabbit blood

---

[3] Meltzer, S. J., *Rep. Johns Hopkins Hosp.*, 1900, ix, 135.

[4] The experiments are all specimen instances selected from a number giving the same results.

was taken in Locke's[5]-citrate,—1 part of blood to 4 of the solution, which latter contained 1 per cent of citrate,—and some of the mixture was tubed and set aside. The cells of the remainder were washed twice with Locke's fluid and to some of the cells the original supernatant fluid, a mixture of plasma and Locke's-citrate, was restored, in the original proportion, and tubing done as usual.

After five days in the ice box there was well marked hemolysis of the washed red cells but none of those left undisturbed.

Experiment 3. Red Cells That Break Down Rapidly when Shaken in Locke's Solution Remain Intact in Plasma-Locke's-Citrate. The Plasma is the Protective Agent.—Some blood was taken from two normal sheep, two rabbits, two dogs, and two men into Ringer-citrate, in the usual 1:4 proportion. Half of each mixture was washed twice with Ringer's solution and the original volume restored with it. There was no hemolysis. The washed and unwashed specimens of blood were now placed in shaking tubes of uniform size. The same considerable air space was left in each tube and duplicate preparations were made. All were now shaken in a machine for 15 minutes, the tubes centrifugalized, and the amount of hemolysis read.

The washed human bloods shaken in Ringer's solution showed each a trace of hemolysis, the two sheep bloods considerably more, an amount which may be indicated by +, the rabbit bloods + + +, and the dog bloods + + + and + + + +, respectively. There was no hemolysis in any of the tubes containing plasma-Ringer's-citrate. The duplicate preparations confirmed these results.

As a corollary to this experiment, tests were made to see whether Ringer's-citrate without plasma had a protective action. Washed cells shaken in it went to pieces with the same rapidity as in ordinary Ringer's solution.

It is evident that the handling of red cells in salt solution, even to the small extent necessary to wash them, may be very injurious. Much of the injury is immediate and mechanical in character. Plasma has a notable influence to prevent it.

Protection.

From these observations it is plain that if washed red cells are to be properly preserved they must be protected during washing. Plasma obviously cannot be used for this purpose. Some simple agent is needed. And this was found in gelatin. $\frac{1}{8}$ to $\frac{1}{4}$ per cent of gelatin in Locke's solution protects cells absolutely against injury during washing, and even during prolonged shaking.

[5] The Locke's solution referred to here and elsewhere is Locke's modification of Ringer's fluid, but without any sugar: 9.2 gm. sodium chloride, 0.05 gm. sodium bicarbonate, 0.1 gm. potassium chloride, 0.1 gm. calcium chloride in 1,000 cc. of water.

Experiment 4. The Protective Influence of Gelatin.—Dog blood was taken in Locke's-citrate as usual (1 part to 4), distributed in equal quantity in eight tubes, and centrifugalized at high speed. From all except one of the tubes the supernatant fluid was now pipetted off as completely as possible, by means of a capillary pipette. The original volume was restored to six tubes with Locke's solution containing graded amounts of gelatin, from $\frac{1}{2}$ per cent to $\frac{1}{64}$ per cent, while to the seventh tube ordinary Locke's solution was added. All were now stoppered, shaken in a machine for 15 minutes, centrifugalized, and the hemolysis was noted. Shaking was then renewed for 15 minutes, and the tubes were placed in the ice box and examined after 3 days. The results are given in Table I.

TABLE I.

Hemolysis.

Time of shaking.	Locke's solution	$+\frac{1}{64}$ per cent gelatin.	$+\frac{1}{32}$ per cent gelatin.	$+\frac{1}{16}$ per cent gelatin.	$+\frac{1}{8}$ per cent gelatin.	$+\frac{1}{4}$ per cent gelatin.	$+\frac{1}{2}$ per cent gelatin.	Citrated blood.
min.								
15........	+++	+++	+++	+−	0	0	0	0
30........	+++	+++	+++	+−	0	0	0	0

Many subsequent experiments confirm this one. The protection afforded by the addition of $\frac{1}{8}$ per cent of gelatin to Locke's solution is for practical purposes perfect, and we have employed it regularly when blood was to be washed. Cells of the sheep, dog, and rabbit thus protected last days longer than when handled in ordinary Locke's solution.

Specific Differences in Fragility.

Protection is especially needed in the case of the red cells of the dog. Ottenberg, Kaliski, and Friedman[6] in some experiments on the normal hemagglutinins of this animal found that erythrocytes washed and placed in salt solution broke down too rapidly to be used. They were obliged to employ cells suspended in their own serum. Usually we have noted an abundant hemolysis within a few hours of dog cells washed after the ordinary methods. But if gelatin-Locke's be the washing medium, they may remain intact for several days. The erythrocytes from different dogs show marked differences in their period of survival.

[6] Ottenberg, R., Kaliski, D. J., and Friedman, S. S., *Jour. Med. Research*, 1913, xxviii, 141.

It has proved interesting to compare the resistance to mechanical injury (shaking) of the cells of different species. The results obtained in Experiment 3 express a general rule. Human cells have by far the greatest resistance. In their case, as we have repeatedly found, washing with Locke's solution after the ordinary method entails no perceptible injury, the cells remaining unhemolyzed as long as when gelatin-Locke's is used. Sheep cells come next in point of endurance. But washing in ordinary Locke's solution injures them somewhat, and it affects much more considerably rabbit cells and dog cells. All of these require protection by gelatin. An absolute scale of the fragility of bloods is difficult to prepare because marked variations are observed with the cells of different individuals and with the length of time that shaking is carried on. Dog blood at first breaks down far more than rabbit blood, but as shaking is continued the latter shows the greater destruction. In order properly to illustrate the findings, curves should be constructed like those that Smith and Brown[7] have used to record the percentages of erythrocytes breaking down in salt solutions of graduated hypotonicity.

Resistance to Shaking versus Resistance to Hypotonic Solutions.

Has the resistance of erythrocytes to mechanical injury any relation to their behavior in hypotonic salt solution?

TABLE II.

	Sheep C.	Sheep M. A.	Dog B.	Dog Dal.
Minimum resistance in salt solution =	0.76 per cent.	0.70 per cent.	0.58 per cent.	0.50 per cent.
Hemolysis on shaking.	Tr.	++	++++	++++

Experiment 5. The Resistance of Erythrocytes to Hypotonic Salt Solution, and to Shaking Vary Independently.—Blood was taken from two dogs often bled previously and from two sheep immunized against a bacterium and also bled often. 15 cc. of each blood were allowed to flow into an equal amount of a solution containing 4 per cent of sodium citrate, 0.6 per cent of sodium chloride, and the other salts of Locke's solution in the usual amount.

[7] Smith, T., and Brown, H. R., *Jour. Med. Research*, 1906, xv, 425.

Each specimen was divided into two equal parts; these were washed twice with Locke's fluid, and one was made up with it as a 10 per cent suspension for shaking, while the other, in thick suspension, was used for tests of resistance to salt solution. Shaking was carried on for 10 minutes and the tubes were centrifugalized and read. Two drops of the thick suspension were added to a number of tubes containing 3 cc. of hypotonic sodium chloride solution. The tubes of hypotonic salt solution were so prepared that each differed from the next by 0.02 per cent of sodium chloride. Readings were taken from these after 3 hours and again after 12 hours.

The results are shown in Table II. The percentages of salt solution recorded are those giving the faintest trace of hemolysis.

In this experiment the cells of the sheep were far less resistant to hypotonic salt solution than those of the dogs, but to shaking they exhibited much the greater resistance. The same phenomenon was evident in the case of individuals of one species (Sheep C. and M. A.). This inverse relationship between the two resistances does not always hold. Human erythrocytes, as we have found, are very resistant to shaking and quite resistant to hypotonic solutions. Hamburger[8] has pointed out that resistance to hypotonic salt solution is the result of many factors, and Rywosch[9] and Rous[10] respectively have proved that it is independent of resistance to chemical hemolysins and to a specific serum hemolysin. The fact that it is also independent of resistance to mechanical injury shows that the term fragility, so often used in connection with it, is a misnomer. Resistance to hypotonic solution is in no real sense an index to the fragility of red cells. A clinical investigation of this fragility as determined by shaking experiments might be not without importance.

Protection versus Preservation.

The injury sustained by cells washed and kept in salt solution is one cause for their rapid breaking down, but it is not the only one.

Experiment 6. Locke's Solution Is Injurious to Cells Kept in It after Washing.— Dog blood was taken as usual in citrate and twice washed, part with Locke's

[8] Hamburger, H. J., Osmotischer Druck und Ionenlehre in ihrer Bedeutung für die Physiologie und die Pathologie des Blutes, Berlin, 1912.

[9] Rywosch, D., *Arch. f. Physiol.*, 1906–07, cxvi, 229.

[10] Rous, P., *Jour. Exper. Med.*, 1909, xi, 763.

solution and part with Locke's solution containing $\frac{1}{2}$ per cent of gelatin. The fluid was taken off, as far as possible, by means of a capillary pipette, and the cells were made to $12\frac{1}{2}$ per cent suspension with ordinary Locke's. 1 cc. of each suspension was now added to 3 cc. of the original plasma-Locke's-citrate and to ordinary Locke's solution, respectively. Examination of the tubes after they had been 4 days in the ice box showed that there was no hemolysis of the cells washed in gelatin-Locke's and kept in plasma-Locke's-citrate, and only the faintest trace in the case of those washed in Locke's. Cells kept in Locke's after washing in gelatin-Locke's showed a + hemolysis, and those washed in plain Locke's a ++ hemolysis.

Here the protective action of gelatin is evident. But it is also plain that, whether red cells are protected during washing or not, they break down sooner in Locke's solution than in a mixture of Locke's with plasma and sodium citrate.

Preservation.

The search for a preservative was now begun. The form of the experiments was simple. Blood was taken in Locke's solution containing 1 per cent of sodium citrate, and its cells were twice washed with Locke's containing $\frac{1}{8}$ per cent of gelatin. All possible fluid was then pipetted off, the cells made up in suspension with Locke's, and portions added to the various preservative fluids. These had, for the most part, Locke's solution, without sugar, as a base. We are aware that other solutions, Tyrode's for example, might have proved better; but Locke's fluid has the advantage of simplicity, and some preliminary observations suggested that cells last longer in it than in 0.95 per cent sodium chloride or in ordinary Ringer's fluid. The substances tested for preservative action were all of high purity. Small vials with sterile corks were used as containers, and only enough cells were placed in each to cover the bottom thinly. This was done because it was found that cells allowed to sediment in a thick layer exhibit very slowly the action of the supernatant fluid. So many vials were used in each experiment that it was not practicable to test the sterility of each preparation by means of culture, and instead reliance was placed on duplicate preparations, on repetition of the work, and on the graded character of the tests which ensured graded results except when some technical error was present. The preparations were kept in the dark under aseptic conditions at a temperature of 1° to 3°C. At first the

appearance of hemolysis was taken as the limit of the period of survival of the cells; but as the work progressed and more precise evidence was needed, it was obtained by transfusing the kept cells in bulk. The transfusion experiments are dealt with in the second part of this paper.

The action of sodium citrate was tested out because of the results with plasma-Locke's-citrate. But though a 1 per cent solution of it in Locke's fluid proved better for dog and rabbit corpuscles than plain Locke's solution, it had not sufficient preservative action for practical purposes.

The first step toward preservation of the cells seemed to be to obtain conditions that would not be deleterious to them. In salt solutions such as Ringer's and Locke's the conditions are far from natural. Red cells placed in them undergo a change of form, becoming spherical (Hamburger).[11] Weidenreich[12] attributes this to the absence of colloids. So tests were begun with Locke's solution to which colloids of various sorts had been added. Gelatin was first employed. It has no preservative action, but, on the contrary, in the amount of $1\frac{1}{2}$ per cent, which restores the normal shape to the cell (Weidenreich), it causes a gradual hemolysis and browning of the blood pigment. Washed agar, soluble starch, plain starch, and dextrin were also tried, but only the last proved useful, and that only for dog blood. The fluid pressed from serum coagulated by heat, and serum water made up to isotonicity with the salts of Locke's gave poor results.

A number of observers[13] have shown that the red cells are almost totally impermeable to sugars in contrast with other crystalloid substances. Solutions of sugar, then, not inconceivably might act like colloids in their effect on the shape of the cells. Sugars dissolved in Locke's fluid were accordingly tried and in them red cells were found to be preserved intact for a remarkable length of time. Dextrose and saccharose had the most marked action, but levulose, maltose, and lactose were not far behind. The cells still remained spherical, however.

[11] Hamburger, H. J., Osmotischer Druck und Ionenlehre in ihrer Bedeutung für die Physiologie und die Pathologie des Blutes, Berlin, 1912.

[12] Weidenreich, F., *Folia Haematol.*, 1905, ii, 95.

[13] Hedin, Hamburger, Kozawa.

The best results were obtained with mixtures of Locke's solution and isotonic solutions of the sugars in water.[14] Isotonic mixtures are better than hypertonic, though slight variations in tonicity are well borne. The small amount of sugar present in Locke's solution, when it is made up after the usual formula, is far below that which exerts a preservative influence. In isotonic sugar solutions alone erythrocytes keep fairly well, but they sediment in a firm layer which can hardly be suspended without hemolysis. The sugar solution and Locke's solution to be mixed together must be autoclaved separately, because in mixtures of them the sugar caramelizes during the autoclaving.

Optimum Preservative Fluids.

For the cells of each species a special preservative mixture is required. In our experience the optimum preservative for the washed cells of the sheep is one made by adding to Locke's solution sufficient saccharose in isotonic, watery solution to give an ultimate concentration of 2.8 per cent of saccharose. Cells twice washed in gelatin-Locke's remain unhemolyzed in this sugar medium for at least 3 or 4 weeks; and even after 2 months hemolysis is exceedingly slight. As is well known, the cells begin to break down within 3 or 4 days when washed and kept in Locke's solution in the ordinary way. For their proper preservation, it is essential that they be washed in an abundance of fluid, since a trace of plasma soon leads to hemolysis. The properly washed and kept cells retain their color; they take up and give off oxygen readily; and though they sediment into a rather firm layer they are easily made into a uniform suspension which passes readily through a filter paper. The cells in such a suspension are discrete, not stuck together. They will withstand repeated washings, but after washing they break down somewhat sooner in Locke's solution than do fresh cells. Cells kept for 3 weeks and for a month have been used for the Wassermann reaction and compared with cells freshly obtained from the same sheep. They had the same hemolytic titer and gave identical results in the reaction.[15]

[14] Saccharose 10.3 per cent, dextrose 5.4 per cent.

[15] For these tests we are indebted to Dr. Russell L. Cecil.

Dextrose is a fairly good preservative for washed sheep cells and human cells. But the latter are best kept in a saccharose-Locke's mixture containing about 5 per cent of the sugar (4.9 per cent). We have thus preserved them for 4 weeks without any hemolysis in the supernatant fluid or on washing. They are easily suspended, and like sheep cells take up and give off oxygen readily. Cells of both sorts begin to break down in about 10 days in a plasma-Locke's-citrate mixture.

Washed rabbit cells remain unhemolyzed longest in plasma-Locke's-citrate, slightly over 2 weeks as a rule. In a mixture of Locke's with glucose and saccharose, containing 3 and 6 per cent of the sugars, respectively, they stay intact from 9 to 12 days.

Dog cells are most difficult to keep. They are very frail, and hemolyze rapidly in their own plasma. In a saccharose-dextrin-Locke's fluid containing 1.6 to 2.7 per cent of the sugar and 2 per cent of dextrin, they show no breaking down for from 5 to 12 days, when they have been thoroughly washed with gelatin-Locke's before being placed in this medium. Preservation for so long a time must be regarded as notable for elements which under ordinary circumstances, washed and kept in Locke's or sodium chloride solution, begin to hemolyze at once. 2 per cent of dextrin added to the Locke's-sugar mixture aids the preservation of dog cells; but it cannot be regarded as uninjurious, since in amounts of 10 per cent it causes a gradual browning of the blood pigment to methemoglobin. This alteration is not evident with 2 per cent dextrin.

We are inclined to attribute the hemolysis that eventually occurs in the optimum preservatives to autolysis within the substance of the cells. That it is not due to the action of the preservatives themselves is well shown by comparing the results with cells allowed to settle in these fluids and cells agitated each day. In such an experiment gelatin must be present to prevent mechanical injury. If the fluid used as a preservative is harmful the cells brought in daily contact with it by agitation break down much sooner than those left to sediment. This is what happens in Locke's solution, for example. But with fluids such as the saccharose-Locke's that is optimum for washed sheep cells, the sedimented and agitated cells show the same gradual breaking down.

Concomitant Factors.

There are many factors besides the character of the preservative which conceivably may affect the period of survival of the cells. Sedimented cells lose their oxygen within a few days; they are exposed to a possible digestive action of the leukocytic pellicle, and to possible injury of the living erythrocytes by contact with dead cells scattered in the mass. Cells that are stirred remain bright and are not exposed to the dangers mentioned. But in experiments specifically directed to these points, we have found that red cells deprived of oxygen last no longer than those well supplied with it, that the leukocytic pellicle of normal blood does not cause hemolysis of the erythrocytes, and that contact injury of living cells by the dead can be disregarded. *A priori* one might suppose that cells would keep best in a very small quantity of preservative fluid, since in this there would be less loss of their diffusible substances. But tests in which the cells were stirred daily and allowed to settle through long and short columns of fluid, have failed to show that this has any importance. It is our practice to place the washed cells in five or six times their bulk of preservative fluid and allow them to remain in sediment until wanted.

Preservatives Are Not Protectives.

If the preservative solutions protect the cells against mechanical injury, gelatin can be dispensed with and a single solution used throughout.

Experiment 7. Dextrose and Dextrin Are Not Protectives.—Dog blood was caught in Locke's-citrate as usual and the mixture distributed in equal amount in 5 centrifuge tubes. The cells of 4 were washed twice with ordinary Locke's solution, Locke's containing 5 per cent of dextrin (Merck), 3 per cent of dextrose, and $1\frac{1}{2}$ per cent of gelatin, respectively, and were suspended to the original blood-citrate bulk in these fluids. With the 5th tube the form of washing was twice gone through, using the original fluid. All were now corked, shaken for 15 minutes, and centrifugalized. There was no hemolysis in the tubes containing gelatin-Locke's and plasma-Locke's-citrate, but in the others it was abundant and of about the same amount in all.

Plasma possesses both protective and preservative qualities, but it is, of course, no simple fluid.

The Preservation of Leukocytes.

Some tests were made to determine whether leukocytes require the same protective and preservative media as the red cells. An aleuronat exudate of the dog containing many large mononuclear cells was washed, half in gelatin-Locke's, half in ordinary Locke's solution, and portions were distributed in various fluids for preservation. As a control, the red cells of the same dog were similarly treated. After 1 week in the cold, all were washed, this time in ordinary salt solution, and the ability of the mononuclear cells to take up rat erythrocytes was tested, with fresh dog serum as complement. Only those cells which had been placed for keeping in the original citrated plasma now showed phagocytosis, and this was independent of whether gelatin had been present in the original wash fluid. Mononuclears kept in Locke's solution, and in the sugar-Locke's, and the sugar-Locke's-dextrin mixtures most favorable to the red cells, failed entirely to ingest the rat corpuscles.

The Preservation of Unwashed Red Cells.

The results with red cells gave some ground for the hope that the erythrocytes of blood received directly into a medium preservative for the washed cells and thus kept, would remain unhemolyzed longer than under the usual conditions. Experiments along this line were undertaken. It was necessary, of course, to use some anticoagulant, and for this purpose sodium citrate was employed. Considerable quantities of blood were taken, distributed with the preservative mixtures in large test-tubes, and kept in the cold. To estimate hemolysis not only was the color of the supernatant fluid noted but the cells were stirred up in $\frac{1}{8}$ per cent gelatin-Locke's and centrifugalized. This frequently brought to light a marked hemolysis of which there had been no trace when the cells lay in sediment. Our freezing point determinations showed that a watery solution of sodium citrate containing 3.8 per cent of the salt is isotonic with 0.95 per cent sodium chloride. Tests were first carried out for an optimum blood-citrate mixture.

Experiment 8. The Optimum Blood-Citrate Mixture for Rabbit Cells.—The blood of two normal rabbits, X and Y, was taken in portions of 3 cc. into sodium

TABLE III.

Hemolysis.

Mixture.	Rabbit X. 26 days.		Rabbit Y. 34 days.		Rabbit X. 34 days.		Rabbit Y. 47 days.	
$1\frac{1}{2}$ cc. blood +	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.
$\frac{1}{20}$ cc. 10% sodium citrate...........	++	+++++	Clotted.		++		Clotted.	
$\frac{3}{16}$ " 10 " " "	++−	++++	++−	++−	++−		++−	++++
$\frac{3}{16}$ " 7.6 " " "	++−	++++	+	++−	++−		+−	++
$\frac{3}{8}$ " 7.6 " " "	++−	+++	Tr.	++−	++−		Tr.	++
$\frac{3}{8}$ " 3.8 " " "	++−	++	+	Tr.	++−		+−	+
1 " 3.8 " " "	+	+	Ft. Tr.	"	+		0	Tr.
$1\frac{1}{2}$ " 3.8 " " "	Tr.	+−	0	Ftest. Tr.	+		0	"

citrate solutions of various concentration and amount. Each mixture was divided into two equal portions and tubed. After many days one tube of each sort was tested for hemolysis, and still later (after 47 days, all told, in the case of Y) the second was examined. The color of the supernatant fluid was recorded and also the amount of hemolysis when the cells were suspended in 6 cc. of $\frac{1}{8}$ per cent gelatin-Locke's and centrifugalized.

The results are shown in Table III.

As this experiment shows, the preservation of the erythrocytes of citrated rabbit blood is much influenced by the amount and concentration of the citrate. We have repeatedly found that the best results are obtained, not with the smallest amount of citrate that will prevent clotting, but with equal parts of blood and isotonic citrate solution. The same amount of a somewhat hypotonic solution (2.5 per cent citrate) also gives good results. With human blood, on the other hand, the proportion and concentration of the citrate seem to have little influence. With sheep and dog blood no quantitative tests have been made.

As Experiment 8 shows, when rabbit blood is mixed with the right amount of citrate its cells remain intact for a long time. The addition of sugars further increases the preservation and the real length of life of the cells to a slight extent, as our transfusion experiments have shown. Sheep cells keep no longer in the presence of sugar. But for human blood cells, it has a remarkable preservative effect.

Experiment 9. The Preservative Action of Sugars on the Cells of Citrated Human Blood.—20 cc. of two human bloods were taken, that from X in an equal bulk of $2\frac{1}{2}$ per cent citrate in water, that from Y in a citrate-salt solution containing 2 per cent of citrate, 0.3 per cent of sodium chloride, and the other salts of Locke's in the same relative proportion. To equal amounts of the blood-citrate mixtures, Locke's solution and isotonic saccharose and dextrose solutions, respectively, were added. One tube of each of these preparations was examined after 13 days and another after 20 days. The sedimented corpuscles were tested for concealed hemolysis by suspending them in $\frac{1}{8}$ per cent gelatin-Locke's and immediately centrifugalizing. The results are given in Table IV.

Experiment 10. The Preservative Action of Sugars on the Cells of Citrated Human Blood.—Three human bloods were taken in various amounts of citrate, as in the case of Experiment 8 with rabbit bloods. To one of the mixtures which contained 3 parts of blood and 2 parts of isotonic (3.8 per cent) citrate solution, 5 parts of isotonic dextrose solution were added. After 4 weeks all the preparations were examined as usual, the cells being suspended in gelatin-Locke's and centrifugal-

TABLE IV.

Hemolysis.

Mixture	Blood X. 13 days.		Blood Y. 13 days.		Blood X. 20 days.		Blood Y. 20 days.	
3 parts citrated blood +	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid	Wash fluid.
0	+−	+−	Tr.	+−			Tr.	+
1 part Locke's.	Tr.	+−	+−	+−	Tr.	++−	+	+
3 " "	+−	+−	++−	++−	++−	++	++−	++
7 " "	++−	++	+++	+++	++	++++	++	+++
1 " saccharose.	0	Tr.	0	+−	Ft. Tr.	+−	Ftest. Tr.	+
3 " "	0	Ft. Tr.	0	+−	0	Tr.	0	+−
5 " " 2 " Locke's.	Ft. Tr.	" "	0	+−	0	Tr.	0	+−
1 " dextrose.	0	" "	0	Ftest. Tr.	Ft. Tr.	0	Tr.	Tr.
3 " "	0	0	0	0	" "	0	Ft. Tr.	0
5 " " 2 " Locke's.	0	0	0	0	" "	0	+	+−

ized. There was no hemolysis of those kept with dextrose, but those of the citrate mixtures all showed a marked breaking down, independent apparently of the amount of citrate present.

Other experiments confirm these results. It is safe to say that the red cells of normal human beings can be kept intact for nearly or quite 4 weeks, when 3 parts of the blood are taken directly in a mixture of 2 parts of isotonic sodium citrate and 5 parts of isotonic dextrose solution. With citrate alone, in any quantity, hemolysis is well marked in less than 2 weeks.

Locke's Solution Is Injurious.

The action of Locke's solution to cause hemolysis, which is so plain in the case of washed cells kept in it, is no less evident when this solution is added to citrated blood. Experiment 9 illustrates this fact for human blood.

Experiment 11. The Injurious Effect of Locke's Solution on the Cells of Citrated Rabbit Blood.—The blood of two rabbits, X and Y, was taken in an isotonic citrate solution, and to portions of the mixtures, Locke's solution and an isotonic watery solution of saccharose, respectively, were added. The tubes were examined for hemolysis after many days had elapsed (Table V).

TABLE V.

Hemolysis.

Mixture.	Rabbit X. 26 days.		Rabbit Y. 34 days.		Rabbit X. 34 days.		Rabbit Y. 47 days.	
3 cc. blood + 2 cc. citrate +	Super-natant fluid.	Wash fluid.	Super-natant fluid.	Wash fluid.	Super-natant fluid.	Wash fluid.	Super-natant fluid.	Wash fluid.
0	+	+	Ft. Tr.	Tr.	+		0	Tr.
3⅓ cc. Locke's.	++++	+−	+++	"	++++		+++	+
3⅓ cc. saccharose.	+	Tr.	0	0	+		Ft. Tr.	0

The greater the amount of Locke's solution mixed with the citrated blood the greater is the destruction of the corpuscles (Experiment 9). But as in the case of washed cells, the injurious action of the Locke's fluid can be completely prevented by means of a sugar.

Experiment 12. The Effect of Saccharose To Prevent the Injurious Action of Locke's Solution on Rabbit Corpuscles.—Rabbit blood was taken into isotonic citrate and to portions of the mixture Locke's solution, isotonic saccharose solution, and combinations of the two were added. After many days the tubes were examined for hemolysis in the usual way (Table VI).

TABLE VI.

Hemolysis.

Mixture.	Percentage in ultimate mixture.				Rabbit X. 26 days.		Rabbit Y. 34 days.	
3 cc. blood + 2 cc. citrate +	Blood.	Citrate.	Sugar.	Locke's.	Super-natant fluid.	Wash fluid.	Super-natant fluid.	Wash fluid.
0	60	40	—	—	+	+	Ft. Tr.	Tr.
3⅓ cc. saccharose.	36	24	40	—	+	Tr.	0	0
3⅓ cc. Locke's.	36	24	—	40	++++	+−	+++	Tr.
8½ cc. Locke's. 9½ cc. saccharose.	13	9	41	37	+	Ft. Tr.	0	0
17 cc. Locke's. 19 cc. saccharose.	7	5	46	42	Tr.	0	0	Tr.

A similar action of dextrose is indicated in Experiment 9 with citrated human blood.

SUMMARY.

The erythrocytes of some species are much damaged when handled in salt solutions, as in washing with the centrifuge after the ordinary method. The injury is mechanical in character. It may express itself in hemolysis only after the cells have been kept for some days. It is greatest in the case of dog corpuscles, and well marked with sheep and rabbit cells. The fragility of the red cells, as indicated by washing or shaking them in salt solution is different, not only for different species, but for different individuals. It varies independently of the resistance to hypotonic solutions.

The protection of fragile erythrocytes during washing is essential if they are to be preserved *in vitro* for any considerable time. The addition of a little gelatin ($\frac{1}{8}$ per cent) to the wash fluid suffices for this purpose, and by its use the period of survival in salt solutions of washed rabbit, sheep, and dog cells is greatly prolonged. Plasma, like gelatin, has marked protective properties.

Though gelatin acts as a protective for red cells it is not preservative of them in the real sense. Cells do not last longer when it is added to the fluids in which they are kept. Locke's solution, though better probably than Ringer's solution, or a sodium chloride solution, as a medium in which to keep red cells, is ultimately harmful. The addition of innocuous colloids does not improve it. But the sugars, especially dextrose and saccharose, have a remarkable power to prevent its injurious action, and they possess, in addition, preservative qualities. Cells washed in gelatin-Locke's and placed in a mixture of Locke's solution with an isotonic, watery solution of a sugar remain intact for a long time,—nearly 2 months in the case of sheep cells. The kept cells go easily into suspension free of clumps, they pass readily through paper filters, take up and give off oxygen, and when used for the Wassermann reaction behave exactly as do fresh cells of the same individual. The best preservative solutions are approximately isotonic with the blood serum. If the cells are to be much handled gelatin should be present, for the sugars do not protect against mechanical injury.

Different preservative mixtures are required for the cells of different species. Dog cells last longest in fluids containing dextrin as well as a sugar. The mixture best for red cells is not necessarily best for leukocytes.

A simple and practical method of keeping rabbit and human erythrocytes is in citrated whole blood to which sugar solution is added. In citrated blood, as such, human red cells tend to break down rather rapidly, no matter what the proportion of citrate. Hemolysis is well marked after little more than a week. But in a mixture of 3 parts of human blood, 2 parts of isotonic citrate solution (3.8 per cent sodium citrate in water), and 5 parts of isotonic dextrose solution (5.4 per cent dextrose in water), the cells remain intact for about 4 weeks. Rabbit red cells can be kept for more than 3 weeks in citrated blood; and the addition of sugar lengthens the preservation only a little. The results differ strikingly with the amount of citrate employed. Hemolysis occurs relatively early when the smallest quantity is used that will prevent clotting. The optimum mixture has 3 parts of rabbit blood to 2 of isotonic citrate solution.

In the second part of this paper experiments are detailed which prove that cells preserved by the methods here recorded function excellently when reintroduced into the body.

DISCUSSION.

Our findings show that in experiments with cells washed in salt solutions there is a large source of possible error in injury done during washing. That it has been so long overlooked by investigators is probably due to the fact that mechanical injury does not alter hemolytic titer, and furthermore that such injury often manifests itself only after the cells have been kept for several days. The reasons for the injury in salt solution and for the protective action of gelatin are not clear. We were led to employ the latter through some observations of Weidenreich,[16] who showed that red cells placed in salt solution containing gelatin do not become spherical as in ordinary salt solution but retain their normal shape. From this he concluded that the shape of the cell is determined not only by the osmotic pressure of the surrounding fluid, but by its molecular force (*Molecularkraft*), as determined through its content in colloidal substances. But the amount of gelatin which will confer protection in salt solution is far too little appreciably to alter the molecular force, or, as we have found, to preserve the normal shape of the cell. For this latter effect, at least

[16] Weidenreich, F., *Folia Haematol.*, 1905, ii, 95.

as much as the $1\frac{1}{2}$ per cent of gelatin, recommended by Weidenreich, is necessary. In Locke's solution containing $\frac{1}{8}$ per cent of gelatin the cells are spherical. In the preservative sugar-Locke's solutions also the cells are spherical. It is interesting that cells kept for many days in such distorted condition should retain their usefulness for the body.

The ability of plasma to protect red cells against mechanical injury may explain to some extent the relatively long survival of the latter in the circulation. A clinical investigation of the mechanical fragility of red cells as determined by shaking experiments might yield results of value.

The preservative action of the sugars on red cells kept *in vitro* is largely dependent, when Locke's solution is present, on a prevention of injury from this latter. That Locke's solution should fail to be a physiological medium for red cells is scarcely surprising. Much recent work has gone to show that for the cells of different organs, different solutions are physiological. But Locke's solution is not merely lacking in some constituent needed by the red cells. It is actively injurious. This is well seen in the experiments with citrated blood, as also in the action of sugars to prevent the injury. The further preservative action of the sugars is perhaps referable to their ability to retard proteolytic digestion;[17] and the peculiarity that it is effective only in the case of certain red cells, to the fact that the erythrocytes of different species have a somewhat different permeability for sugars.[18]

Sheep cells washed and kept according to our methods are as suitable for the Wassermann reaction as fresh cells. But considerable manipulation of them is required. Formalization of the defibrinated blood, as practiced by Armand-Delille and Launoy,[19] and by Bernstein and Kaliski,[20] would seem more practical. The blood-citrate-saccharose mixture should prove useful for the preservation of rabbit and human corpuscles for culture media.

[17] Ogáta, M., *Arch. f. Hyg.*, 1885, iii, 204.

[18] Kozawa, S., *Biochem. Ztschr.*, 1914, lx, 146, 231.

[19] Armand-Delille, P., and Launoy, L., *Ann. de l'Inst. Pasteur*, 1911, xxv, 222.

[20] Bernstein, E. P., and Kaliski, D. J., *Ztschr. f. Immunitätsforsch., Orig.*, 1912, xiii, 490.

THE PRESERVATION OF LIVING RED BLOOD CELLS IN VITRO.

II. The Transfusion of Kept Cells.

By PEYTON ROUS, M.D., and J. R. TURNER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 15, 1915.)

In the foregoing paper methods are described whereby red blood cells may be kept intact for long periods *in vitro*. It has remained to determine whether cells kept according to these methods are alive in the sense that they are capable of functioning in the animal body. This can be told by transfusion of the kept cells in bulk, with appropriate control. We have performed many such experiments, using rabbits.

The cells for preservation were obtained by bleeding etherized rabbits from the carotid or aorta into a Locke's-citrate mixture or an isotonic solution of citrate (3.8 per cent) in water. In some instances, the cells were washed, with gelatin-Locke's ($\frac{1}{8}$ per cent gelatin), and placed in a preservative solution; in others they were kept, without washing, in the original mixture of citrate and blood, or in citrate and blood plus a sugar. When they were to be used the supernatant fluid was pipetted away; and sometimes the sediment was suspended directly in Locke's solution to the original blood bulk and used for injection. More often it was washed once or twice with gelatin-Locke's and then suspended in ordinary Locke's. The suspension was filtered through two thicknesses of gauze; warmed to body temperature; and introduced under slight pressure into the ear vein of a rabbit which had just been bled.

The rabbits which furnished and received the cells were selected from a number tested against one another to rule out the presence of iso-agglutinins and isolysins. In the rabbit such antibodies are weak and infrequent. None of our results suggest their action. The animals chosen as recipients were bled in large amount from the ear

and the hemoglobin loss was replaced by the transfusion of kept cells. The bleeding and injection were repeated if the amount of kept cells allowed, as in instances when the cells from two rabbits had been preserved for the injection of a single individual. In every case a large proportion of the blood was drawn and replaced. The fate of the transfused cells was followed by blood counts, hemoglobin estimations, direct microscopic observations on the appearance of the blood elements, and by daily tests of the urine for hemoglobin (guaiac and spectroscopic tests) and bile (Gmelin and Bouvais tests). In addition, the percentage of reticulated red cells in circulation was noted in wet preparations stained with cresyl blue. This was done because an increase in the reticulated cells indicates an abnormal activity on the part of the bone marrow, such as would be the result of an increased destruction of the circulating erythrocytes. The rabbit's temperature was taken twice daily and its weight frequently recorded.

Some of the results may be briefly summarized and thus a repetition of data avoided. The bleeding and replacement of blood were always well borne. They took from one-half to three-quarters of an hour, and at the conclusion the animal usually went at once to eating. There was never a suppression of urine. Bile was never found in it and blood only in one instance, in a control experiment. The rabbit was, in this case, injected with cells kept, not in a preservative, but in Locke's solution, and a copious breaking down resulted with hemoglobinemia, hemoglobinuria, and death (Experiment 6).

In the specimen protocols that follow, the amount of blood drawn and replaced is recorded in percentage of the animal's total hemoglobin content. This is not to be confused with the percentage of hemoglobin in the circulating blood as obtained with the hemoglobinometer. It gives a much more precise idea of the proportion of formed elements dealt with, than would the blood volume. As is well known, when bleeding is done, even very rapidly, the last portion of blood contains much fewer cells than the first.[1] In thirteen instances in which we have taken into citrate from 30 to 50 per cent of the total calculated volume of a rabbit's blood and have com-

[1] Boycott, A. E., in Pembrey, M. S., and Ritchie, J., Text-Book of General Pathology, New York and London, 1913, 9.

pared the hemoglobin concentration of the drawn blood with that of the animal originally, it was found to be on the average 17 points lower by the Sahli scale. In general we have noted both the volume and the hemoglobin of the drawn blood, and have calculated from these the amount of cell suspension of known hemoglobin content that should be injected to restore the animal's hemoglobin content to the normal. In some of the early experiments this was not done and replacement was made by volume. According to Boycott and Douglas[2] a rabbit possesses 5.5 cc. of blood for 100 gm. of body weight. We have used this figure with hemoglobin estimations by the Sahli scale in determining the proportion of total blood pigment, and thus of erythrocytes, drawn and replaced. Since only 93 per cent of a rabbit's total hemoglobin is in the circulating blood (Boycott

TABLE I.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.
		per cent
Before	4,890,000	87
3 hrs. after	4,590,000	85
24 " "	4,460,000	85
3 days "	4,510,000	83
6 " "	4,450,000	81

and Douglas), our estimate must be considered as rather less than the actual proportion. When two withdrawals and injections were carried out, allowance was made in the calculations for the mixed character of the second portion of blood withdrawn.

Experiment 1. Transfusion with Washed Cells Kept in a Dextrose-Locke's Solution and Washed before Use.—An animal weighing 1,700 gm. was bled 41 cc., and 41 cc. of a suspension of kept blood cells at once injected. By calculation some 30 per cent of the total hemoglobin was thus taken and replaced. The kept blood had been taken eight days previously, twice washed, and placed in Locke's solution to which $2\frac{1}{2}$ per cent of glucose and $\frac{1}{8}$ per cent of gelatin had been added. It was washed twice just previous to injection and suspended in Locke's solution. This handling brought about no hemolysis (Table I).

[2] Boycott, A. E., and Douglas, C. G., *Jour. Path. and Bacteriol.*, 1909, xiii, 256.

The animal was killed on the sixth day because of an infected ear. The reticulated red cells were not followed in this case. Fresh preparations of the blood failed to show shadows.

Experiment 2. Transfusion with Cells Kept in a Blood-Citrate-Saccharose Mixture and Washed before Use.—For this experiment the erythrocytes of two rabbits were used after they had been kept separately in mixtures of the citrated blood with saccharose for 11 and 12 days, respectively. Three parts of the blood were mixed for keeping with 2 of isotonic citrate and 5 of isotonic saccharose solution. The cells were washed once just before injection and suspended in Locke's solution. Following the injection there was no hemoglobin in the supernatant or wash fluid. The rabbit that acted as recipient was twice bled (40 and 6 cc.) and twice injected. 31 per cent of the animal's hemoglobin was thus replaced (Table II).

TABLE II.

Time before or after transfusion.	Red blood corpuscles.	Hemo-globin.	Reticulated cells.	Weight.	Urine.	Temper-ature.
		per cent		*gm.*	*cc.*	*°F.*
1 day before	6,500,000	91	25 in 500	2,025	65	102
Just before	6,100,000	90	19 " "		38?	102
3 hrs. after	6,500,000	90				
1 day "	6,640,000	87	24 " "		150	102.6
2 days "	6,540,000	86	14 " "		80	101.9
3 " "	6,540,000	83	12 " "	2,050	150	101.9
4 " "	6,840,000	91	13 " "		180	101.6
5 " "	6,420,000	85	11 " "		353	101.9
7 " "	6,730,000	89	9 " "		110	101.8
9 " "	6,640,000	90	11 " "	1,875	80	101.9

Fresh preparations of the blood made daily appeared normal throughout. On the third day after the transfusion a slight polychromatophilia was noted.

Experiment 3. Transfusion with Cells Kept in a Blood-Citrate Mixture and Washed before Use.—The blood of two rabbits was taken into citrate as usual (3 parts of blood to 2 parts of citrate) and kept for 11 days. The cells were washed once just prior to injection and suspended in Locke's solution. There was a slight hemolysis in the supernatant and wash fluids. The amount was calculated by comparing the color of these fluids with a laked preparation of the ultimate suspension, and it was found to equal about 0.2 per cent of this suspension. The recipient rabbit was bled 60 cc., or 36 per cent of the total hemoglobin content, and an equivalent amount was injected (Table III).

Throughout in fresh preparations the blood appeared normal. On the second day after transfusion occasional polychromatophilia was noted.

TABLE III.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.	Weight.	Urine.	Temperature.
		per cent		*gm.*	*cc.*	°*F.*
3 days before	6,320,000	87	12 in 500	2,250		101.8
1 day "	6,480,000	85	14 " "		70	101.8
Just "	6,010,000	80	18 " "		35	102.0
5 hrs. after	6,460,000	85				
1 day "	6,380,000	86	9 " "		60	101.4
2 days "	6,820,000	90	16 " "		35	102.2
3 " "	6,820,000	86	11 " "		64	102.2
4 " "	6,440,000	86	6 " "		51	101.9
5 " "	6,560,000	87	8 " "	2,375	140	101.5
7 " "	6,850,000	86	10 " "		250	101.2
9 " "	7,390,000	93	5 " "		172	101.4
11 " "	6,720,000	88	6 " "	2,300	170	102.0

Experiment 4. Transfusion with Cells Kept in a Blood-Citrate-Saccharose Mixture and Suspended Directly in Locke's Solution.—The blood of two rabbits was taken and kept as in Experiment 14 for 13 and 15 days, respectively. The supernatant fluid was pipetted off just prior to injection and the cells suspended in Locke's solution without washing. Two bleedings (of 40 and 50 cc.) were done and two injections. About 64 per cent of the total hemoglobin was thus withdrawn and more than replaced, as the blood examination showed (Table IV).

On the 2nd day after operation there were slight anisocytosis and polychromatophilia.

TABLE IV.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.	Weight.	Urine.	Temperature.
		per cent		*gm.*	*cc.*	°*F.*
1 day before	4,860,000	68	16 in 500	1,800		103.4
Just "	4,790,000	65	20 " "		150	103.2
3 hrs. after	5,670,000	81				
1 day "	5,220,000	76	16 " "		130	102.6
2 days "	5,280,000	73	21 " "		10?	103.0
4 " "	5,360,000	75	19 " "	1,825	170	102.4

Many experiments similar to these were done and with the same general results. Rabbit red blood cells kept for two weeks *in vitro* under suitable conditions can be used with good results to replace the blood lost in a hemorrhage. It is unnecessary to wash the cells which

may be simply suspended in Locke's solution after the preservative mixture is pipetted off. The preservative mixture which we have found best,—blood plus sodium citrate plus an isotonic saccharose solution—cannot be injected with the cells because of its content in citrate, but the small portion of it that remains with the cells after pipetting is not harmful. In blood-citrate mixtures without sugar the cells show some slight hemolysis (Experiment 2) after 2 weeks; and in several instances a slight drop in the cell count and hemoglobin percentage following transfusion, together with a rise in the number of reticulated red cells, has indicated that the kept cells were disappearing from the circulation and that the bone marrow was active in repairing the loss.

TABLE V.

	Time.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.
			per cent	
Rabbit A.				
35 cc. taken of the total 78 cc. of blood	Before operation.	5,500,000	106	2 in 500
	Day after "	2,400,000	48	53 " "
	19 days after "	5,000,000	77	27 " "
Rabbit B.				
45 cc. taken of the total 139 cc. of blood	Before operation.	6,100,000	112	1 " "
	Day after "	3,600,000	65	15 " "
	18 days after "	5,870,000	110	14 " "
Rabbit C.				
42 cc. taken of the total 94 cc. of blood	Before operation.	6,800,000	125	2 " "
	Day after "	3,000,000	54	13 " "
	25 days after "	6,000,000	112	35 " "

In striking contrast to these results are some that were obtained in control experiments.

Experiment 5. Effects of Bleeding Alone.—Two rabbits were bled as usual, but received no injection afterwards. Both died within a few minutes. The calculated blood volume of the animals was 88 and 60 cc., and the bleedings were for 50 and 28 cc., respectively.

Experiment 6. Effects of Bleeding Followed by Injection of Locke's Solution.—Three rabbits were bled and an equivalent amount of Locke's solution was injected intravenously. There was an immediate great drop in hemoglobin percentage and number of red cells. Regeneration was still incomplete after many days (Table V).

Experiment 7. Bleeding Followed by Transfusion of Cells Washed and Kept in Locke's Solution.—A rabbit with a calculated blood volume of 87 cc. was twice bled (44 and 28 cc.) and an equivalent amount of kept cells was introduced. Thus about 56 per cent of the total hemoglobin was replaced. The kept cells had been washed and preserved in Locke's solution for 11 days and they were again washed just previous to injection. At this time some hemolysis was noted. The animal died in less than 24 hours after the injection and on autopsy there were hemoglobinuria, hemoglobinemia, spodogenous spleen, and other findings typical of the breaking down of blood in large quantities.

These control rabbits all fared badly. Evidently the aid rendered by a transfusion of cells kept in a proper preservative is a real one. As Experiments 4 and 5 show, these cells function normally even after they have been kept *in vitro* for 2 weeks. We have performed a number of transfusions with cells kept longer. They remain unhemolyzed for as long as 4 weeks, but by the end of the 3rd week have largely lost their ability to be useful when reintroduced into the body, as shown by the fact that within a few days they disappear from the circulation. This disappearance is unaccompanied by any signs of hemolysis or, indeed, of other derangement. The animal eats well and may gain weight. The anatomical findings in such cases have interest as bearing on methods of blood destruction. Discussion of them will be reserved for another paper. From among the many experiments one will be given here to illustrate the facility with which the body disposes of blood elements no longer useful in the circulation.

Experiment 8. Transfusion with Cells Preserved Too Long in Vitro.—A rabbit weighing 1,925 gm. was bled 54 cc. and transfused with kept cells. 44 per cent (actual) of the total hemoglobin was thus taken and an amount equal to only about 37½ per cent put back. By calculation this should have caused a fall in hemoglobin percentage as determined with the Sahli instrument to 75 per cent after the transfusion, and that indeed was the figure obtained. The cells had been kept for 23 days in a mixture of 3 parts of blood, 2 parts of isotonic citrate solution, and 5 parts of isotonic saccharose solution. For injection they were washed once in gelatin-Locke's solution and suspended in ordinary Locke's. Neither the supernatant nor the wash fluids showed the slightest trace of hemolysis (Table VI).

There was at no time bile or blood in the urine. On the 2nd and 3rd days after the transfusion, there was marked polychromatophilia and some anisocytosis. Otherwise the animal seemed normal. The blood examinations on the 2nd day indicated that nearly all the transfused cells had disappeared from the

TABLE VI.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.	Weight.	Urine.	Temperature.
		per cent		*gm.*	*cc.*	*°F.*
4 days before	5,960,000	78	9 in 500			101.6
3 " "	5,850,000	81	12 " "	1,925	20?	101.8
1 day "					280	
Just "	5,810,000	80	8 " "			102.7
3 hrs. after	5,490,000	75				
1 day "	4,380,000	58	52 " "		100	102.2
2 days "	3,890,000	51	45 " "		120	101.8
3 " "	3,900,000	59	44 " "	1,790	20	101.4

circulation. The rabbit was killed on the 3rd day. The organs appeared normal. The spleen weighed only 0.7 gm. It gave a well marked iron reaction, and showed many phagocyted red cells.

A transfusion such as the above with intact cells kept too long *in vitro* is not helpful, or only indirectly so as supplying the constituents for new red cells; but in our experience it is not harmful.

The Preservation of Human Red Cells.

Human red cells can be preserved *in vitro* much longer than rabbit cells; and there seems little doubt that they could be profitably used for transfusion. Recently Weil[3] has reported upon a number of transfusions with whole citrated human bloods kept in the cold for several days. He employed 1 part of 10 per cent citrate to 10 parts of blood. But Lewisohn[4] has shown that citrate in this amount is dangerous to the organism; and we have found that human blood thus kept with citrate begins to hemolyze in about a week, the hemolysis being more dangerous because it is often completely concealed amid the sedimented corpuscles. Cells kept in a blood-citrate-dextrose mixture, according to the method detailed in the first part of this work,[5] remain

[3] Weil, R., *Jour. Am. Med. Assn.*, 1915, lxiv, 425.

[4] Lewisohn, R., *Surg., Gynec. and Obst.*, 1915, xxi, 37.

[5] Three parts of blood are caught in a mixture of 2 parts of isotonic sodium citrate solution (3.8 per cent) in water, and 5 parts of isotonic dextrose solution (5.4 per cent) in water. The preparation is allowed to remain undisturbed in the ice box until wanted, when the supernatant fluid is drawn off and the sedi-

intact for 4 weeks; and it would seem preferable to use cells thus kept, suspending them for injection in a little salt solution. By this method the plasma is lost; and it is of great importance for some conditions in which transfusion is employed. But in cases of simple exsanguination, as Abel and his coworkers have shown,[6] to furnish corpuscles to the body is sufficient. One might ask whether the slight remnant of plasma, sugar, and citrate remaining with the corpuscles and injected with them would be harmful. Our experiments with rabbits, and recent work on the injection of citrate and concentrated sugar solutions[7] prove this danger to be negligible. The experiments with rabbits show that if blood cells are kept too long to function, but are still intact when restored to the circulation, they are easily disposed of by the body. Tests for iso-agglutinins and hemolysins would be necessary before the transfusion of kept human cells.

SUMMARY.

In order to determine the availability for functional uses of red cells kept *in vitro* by our methods, transfusion experiments have been carried out with rabbits by which a large part of their blood was replaced with kept rabbit cells suspended in Locke's solution. It has been found that erythrocytes preserved in mixtures of blood, sodium citrate, saccharose, and water for 14 days, and used to replace normal blood, will remain in circulation and function so well that the animal shows no disturbance, and the blood count, hemoglobin, and percentage of reticulated red cells remain unvaried. Cells kept for longer periods, though intact and apparently unchanged when transfused, soon leave the circulation. Animals in which this disappear-

ment of cells suspended in Locke's solution. The slight whitish flocculus which is sometimes present above the sediment disappears in the Locke's solution. It has been our practice to filter the suspensions of kept rabbit cells through gauze, previous to use. Needless to say, the preparation must be kept sterile. The sugar and citrate solutions should be autoclaved separately, or the mixture of them can be put through a Berkefeld filter.

[6] Abel, J. J., Rowntree, L. G., and Turner, B. B., *Jour. Pharmacol. and Exper. Therap.*, 1914, v, 625.

[7] Hustin, A., *Ann. et bull. Soc. roy. d. sc. méd. de Bruxelles*, 1914, lxxii, 104. Enriquez. *Presse méd.*, 1914, xxii, 121.

ance of cells is taking place on a large scale, remain healthy save for the progressing anemia. The experiments prove that, in the exsanguinated rabbit at least, transfusions of cells kept for a long time *in vitro* may be used to replace the blood lost, and that when the cells have been kept too long but are still intact they are disposed of without harm. The indications are that kept human cells could be profitably employed in the same way.

THE CAUSE OF RAT-BITE FEVER.

By KENZO FUTAKI, M.D., FTSUMA TAKAKI, M.D., TENJI TANIGUCHI, M.D., and SHIMPACHI OSUMI, M.D.

(*From the Imperial Japanese Institute for Infectious Diseases, Tokyo.*)

Plate 33.

(Received for publication, December 10, 1915.)

Four cases of rat-bite fever have recently come under our observation, and we have been able to make a systematic study of two of them.

The symptoms of the disease, which usually begin after an incubation period of from 10 to 27 days, consist of chills, fever, headache, and malaise. Inflammation is soon observed at the site of the apparently healed bites, with pains in the limbs on the affected side of the body, dark red skin eruptions, and swelling of the lymph glands. The attack with its local manifestations and high fever continues for from 3 to 7 days, the duration varying in individual cases. It alternates with an afebrile interval of 2 or 3 days. All the cases that came under our observation showed this picture, which may be considered typical of rat-bite fever.

Our investigations were conducted in the following manner. On August 9, 1915, when one of the patients was showing active symptoms, a sterile excision was made of a small piece of skin from the arm. This piece of skin showed the typical exanthem described above. The excised tissue was placed under dark-field illumination and examined microscopically, and in it were observed numerous actively moving spirochætæ. The skin tissue and also blood drawn from the patient were inoculated into monkeys, guinea pigs, and white rats. It was possible to infect all the animals inoculated in this manner, and to transmit the disease from them to other animals.

The study with the second patient was even more detailed. During the second attack a swollen lymph gland was punctured, and an India ink preparation made of the exudate, according to the

method of Burri. At the same time, a section of the excised lymph gland was impregnated with silver, according to Levaditi's method. The spirochætæ were identified in both preparations (Fig. 1).

The organism is somewhat larger than *Spirochæta pallida*, but smaller than *Spirochætæ duttoni* and *obermeieri*.

One of the patients was treated with mercury, and the other with salvarsan. Both recovered. In treating these cases we recalled the fact that in recurrent fever, one of the spirochætal diseases which shows a temperature curve similar to that of rat-bite fever, salvarsan has proved effective. Syphilis, also, which resembles rat-bite fever in swelling of the lymph glands and skin eruptions, is improved or cured by salvarsan or mercury.

Hence, not only on the basis of the microscopic findings, but also by comparison with related diseases, we are led to the conclusion that the spirochæta discovered by us is in all probability the cause of rat-bite fever. Further investigations on the subject are in progress, and we reserve the privilege of making more definite statements in the future.

EXPLANATION OF PLATE 33.

Fig. 1. Section of a lymph gland impregnated with silver nitrate according to Levaditi's method. In the center is seen a mass of spirochætæ.

A CONTRIBUTION TO THE BIOLOGY OF PERIPHERAL NERVES IN TRANSPLANTATION.

II. Life of Peripheral Nerves of Mammals in Plasma.

By RAGNVALD INGEBRIGTSEN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research, and the Pathological Institute of the University Clinic, Christiania.*)

Plates 34 to 41.

(Received for publication, July 21, 1915.)

Nageotte[1] has demonstrated that in pieces of peripheral nerves removed from the living animal and kept *in vitro* under certain conditions, the first stages of Wallerian degeneration may occur exactly as in the peripheral part of a divided nerve in the living organism. The phenomena are identical, but the process takes place more quickly *in vitro*. The conditions required for this degeneration *in vitro* are the presence of a solution of sodium chloride with some salt of bivalent metals (calcium, strontium, or magnesium) in suitable proportions, such as Ringer solution.

Nageotte found that the incubation of nerve fibers in isotonic solution of sodium chloride for twenty-four hours left them unaltered, and if they were put in Locke's solution after several days they degenerated exactly as if they were put directly into this fluid. I have observed a typical Wallerian degeneration in nerve fibers incubated in homologous and heterologous serum at body temperature for twenty-four hours, and in this case, as well as in Locke's solution, the degeneration takes place much more quickly than in the organism. One would expect, therefore, a Wallerian degeneration as a result of the incubation of peripheral nerves in plasma, but this did not take place.

[1] Nageotte, J., *Compt. rend. Acad. d. sc.*, 1910, cl, 731; *Compt. rend. Soc. de biol.*, 1910, lxix, 556.

EXPERIMENTAL.

The experiments were performed in the following way. From a living rabbit pieces $\frac{1}{2}$ to $\frac{2}{3}$ cm. long were removed from the sciatic nerve, and the nerve fibers were dissociated in Ringer solution by means of needles. The fibers were put immediately into a drop of plasma on a cover-glass, which was inverted over a hollow slide, sealed with paraffin, and incubated at 38° C. The preparations were observed from day to day under the microscope on a heated table and no change in the fibers was noticed up to the 10th or 12th day after incubation (Figs. 1 and 2). In hardened and stained preparations the nerve fibers had the same appearance on the 6th, 7th, and 8th days as on the 1st day.

I attempted to produce a degeneration in the nerves by adding calcium chloride to the plasma, but the results were the same. One drop of a 1 per cent solution of calcium chloride was added to 5, 7, and 8 drops of plasma, and from each of these mixtures a series of preparations were incubated at 38° C. As a control 1 drop of 1 per cent solution of calcium chloride was added to 5, 7, and 8 drops of serum and to 5, 7, and 8 drops of Ringer solution in which nerve fibers were incubated.

In the nerve fibers from the calcium plasma no degeneration occurred, whereas the nerves from the calcium serum and calcium Ringer solution tubes developed a degeneration in the usual way.

It is well known that pieces of connective tissue, glandular organs, skin, etc., when incubated for some time in plasma give rise to a growth of tissue around the original fragment, in some cases of the same specific cells as those constituting the original piece, but more often built of cells resembling the connective tissue cell type. Accordingly, it was to be expected that in the experiments a growth of tissue would occur originating from the cells of Schwann, but this did not take place. I prepared 80 cultures of nerve fibers removed from the sciatic nerve of living rabbits and in none was growth observed up to the 16th day of incubation.

I then cut the sciatic nerve of a rabbit, closed the wound, and twenty-four hours later removed a piece from the peripheral part. From this piece of nerve which had developed the first faint changes

of the Wallerian degeneration 10 cultures were prepared. No growth was observed from these nerve fibers.

Another series of 32 cultures was prepared, in the usual way with a hanging drop of plasma in hollow slides, from the peripheral part of a divided nerve 2 days after section. There were no changes in the fibers, and no growth was observed up to the 16th day.

Twelve cultures were prepared from the peripheral part of a divided nerve 4 days after section, and there was no growth.

In a series of 36 cultures prepared from the peripheral part of a divided nerve 5 days after section different results were recorded. In 6 of these 36 cultures I observed a growth of characteristic appearance.

After incubating the cultures for 3 and 4 days a number of thin filaments consisting of light-breaking, slightly granular protoplasm were seen growing out from the cut ends of the nerve fibers. In the beginning they have a conic or tapering shape ending in a point in which are seen ameboid movements, by means of which they emerge into the plasma, gain in length, and soon take on a more or less regularly cylindrical appearance measuring in width the same as or slightly less than the individual nerve fibers from which they have grown out. They branch freely, and from the 5th day they assume other characteristics, which are, however, best studied after fixation and staining.

Some of the cultures were hardened in formalin (2 per cent) and subsequently stained in hematoxylin. Others were hardened by means of the Held pyridin method and stained by Cajal's silver nitrate method.

Preparations Stained by Hematoxylin.—(Figs. 3, 4, and 5.) The original piece of nerve is 3 to 8 mm. long and about $\frac{1}{2}$ to 1 mm. wide. In its two ends the individual nerve fibers are easily discernible and display the ordinary appearance of Wallerian degeneration from this stage (that is, on the 5th day). Included in the sheaths of Schwann the following structures are seen: myelin ovoids containing fragments of the axis cylinder, and between 2 ovoids the nuclei of Schwann dislodged into the center of the fiber and embedded in protoplasm. From the cut ends of the individual nerve fibers the newly grown structures enter the plasma. In many of the fibers that give rise to growth, the cross-section of the fiber made by its removal from the

animal has passed close by a nucleus of Schwann, and such a cell marks the end of the old fiber and the beginning of the young one. In only a few of the old fibers a nucleus of Schwann is not present at the end, but in these cases it is found in the fiber only a few microns further in toward the center.

When they reach the plasma, the newly grown protoplasmic structures which form a direct outgrowth from the perinuclear protoplasm (syncytium) of Schwann of the old fibers are slightly fibrillary or granular. They have a tapering shape and a width averaging at their starting point 6 to 8 microns; that is, slightly less than the fibers from which they have grown out. Their tree-like branches are much thinner than the main stem and end in a point or a little bulb, round or oval in shape. In some of the newly grown filaments from distance to distance a well outlined and darkly stained nucleus is seen. These nuclei are oval or fusiform with their longest diameter in the direction of the filament; their shortest (transverse) diameter is ordinarily larger than the filament itself and the presence of the nuclei in the filaments produces varicosities of a corresponding size.

In some of the filaments 3 to 4 nuclei appear in a row one after another embedded in a granular column of protoplasm without any membrane, the whole picture resembling the contents of the sheaths of Schwann in a degenerating nerve 14 to 15 days after section. To complete this likeness we find included in some of the grown out threads small agglomerations of fatty (myelin) globules of different volume from that of minute granules up to or even surpassing the size of a nucleus of Schwann, embedded in varicose accumulations of protoplasm and generally beside a nucleus of Schwann.

In some preparations the filaments after reaching the plasma anastomose between each other, and thus form a real framework-like syncytium (Fig. 4). After 6 days of incubation the newly grown filaments are about 500 microns in length.

Silver-Impregnated Preparations.—The Held pyridin method was found to be the only practicable one for fixing the cultures. It causes no precipitation in the plasma and the subsequent staining by Cajal's silver nitrate method has given good results.

The original fragments of nerves stain dark brown and the surrounding plasma a light yellowish brown. The filaments which have

grown out are slightly darker brown than the plasma and are easily discernible against this background. In no case did the newly grown filaments assume the black, or dark brown color which is characteristic of axis cylinders treated according to this method, which I have demonstrated growing out from pieces of cerebellum and spinal ganglia incubated in plasma.

Morphologically the preparations display the same appearance as the hematoxylin preparations.

The newly grown filaments emerge from the transversely cut ends of the nerve fibers, at the end of which a nucleus of Schwann surrounded by protoplasm is often observed; or such a cell is seen a little further in toward the center of the piece, or in the young fiber a few microns from its base. The filaments are of different sizes, measuring in width from 2 to 3 microns up to 8 to 9 microns at the point where they enter the plasma. From this point they steadily decrease in size and in their course through the plasma they curve and bend in various directions, although as a rule the main direction in the beginning of their growth at least is parallel to the nerve fibers of the original fragment. The filaments are for the most part unevenly shaped and provided with large and small varicosities. The large varicosities include one and sometimes two oval nuclei of the Schwann nucleus type, the small varicosities are formed by accumulation of granular protoplasm. Some of the largest filaments divide into two or three branches which part from the main stem at acute angles, usually leaving a little varicosity of protoplasm at the point of division. The finest branches are provided with minute varicosities and they end in a point or in a little granular protoplasmic bulb.

In a series of 20 cultures prepared from the peripheral part of a divided nerve 6 days after section a growth of filaments was observed in 5. The newly grown structures had the appearance described above (Figs. 6 and 7).

I prepared cultures from the peripheral part of a divided nerve, 8, 10, 13, 15, and 19 days after section. The results of these experiments are shown in Table I.

The following conclusions may be drawn from these experiments. Plasma cultures prepared from normal peripheral nerves give rise

to no proliferation of cells. Plasma cultures prepared from the peripheral part of a divided nerve within the first 4 days after section give rise to no growth. 16 per cent of plasma cultures prepared from the peripheral part of a divided nerve 5 days after section give rise to a proliferation in the plasma of the syncytium of Schwann, and in series of cultures prepared from such a divided nerve in Wallerian degeneration there is a direct ratio between the stage of the Wallerian degeneration counted in days and the percentage of positive cultures as far as the growth of syncytium of Schwann is concerned.

The growth in plasma of the syncytium of Schwann from nerve fibers in a Wallerian degeneration of a more progressed stage, from the

TABLE I.

Cultures of the Peripheral Part of a Divided Nerve in Different Stages of Wallerian Degeneration.

Stage of Wallerian degeneration (No. of days elapsed after section).	Total No. of cultures.	No. of cultures in which growth had occurred.	Percentage of growth.
days			
1, 2, and 4	54	None	0
5	36	6	16.6
6	20	5	25.0
8	20	6	30.0
10	20	8	40.0
13	24	11	45.8
15	21	16	76.0
19	17	14	82.3

13th day for instance, displays minor variations from what has been described concerning the growth from fibers in an earlier stage. The growth is more abundant and the rate of growth is quicker. The fibers are mostly parallel; they are much thinner in their whole length and are far more richly provided with nuclei of the ordinary oval Schwann nucleus type. Around many of these nuclei there is an agglomeration of fatty (myelin) granules embedded in scant protoplasm. The filaments from the later stages of Wallerian degeneration are not so liable to branch, and anastomoses are not seen (Figs. 8, 9, 10, and 11). In Cajal preparations from this stage too, the fibers stain light brown and none of them take the black or dark brown

color which is characteristic of the axis cylinders. Besides the filaments a migration and growth of ordinary connective tissue cells (Fig. 3) are found in many of the cultures.

I should mention here the results of my experiments on the cultivation of pieces of the central nervous system and spinal ganglia of young mammals. In 1913[2] I observed that the brains of chick embryos, of cats 6 weeks old, of rabbits 2 months old, and of dogs 3 weeks old, when cultivated in plasma developed long (1.25 mm.) protoplasmic filaments, which were studied mainly from living preparations and were interpreted as nerve fibers. Such structures developed also from spinal ganglia of rabbits 7 months old and from the spinal cord of cats 6 weeks old. In a later communication,[3] after having succeeded in staining the preparations by means of Cajal silver impregnation, I stated in confirmation of my preliminary report, that nerve fibers grow out from pieces of cerebellum and spinal ganglia of young cats and guinea pigs when cultivated in plasma, and that these nerve fibers did not anastomose, and extended into the plasma unaccompanied by structures of any kind.

Certain other phenomena of growth were also observed by means of the silver impregnation which made possible a discrimination between two different kind of filaments, one of which was nerve fibers and the other probably neuroglia. The proof that nerve fibers grew into the plasma was based upon the direct and continual extension of the black silver-stained nerve fibers of the original pieces into the plasma, where they formed evenly cylindrical or uneven and varicose, mostly branching filaments, which were never granular and did not anastomose.

Besides these fibers an abundant growth of syncytial protoplasm was observed. In unstained living preparations it is difficult if not impossible to tell whether a filament is a nerve fiber or a neuroglia fiber, the difference between them being rather insignificant aside from the anastomoses, which, judging from the stained specimens, frequently occur between the neuroglia fibers but never between the nerve fibers. In silver-stained specimens, however, there is a marked

[2] Ingebrigtsen, R., *Jour. Exper. Med.*, 1913, xvii, 182.

[3] Ingebrigtsen, R., *Jour. Exper. Med.*, 1913, xviii, 412.

difference between the grown out nerve fibers and the neuroglia syncytium.

As shown in Fig. 12, the filaments supposed to be neuroglia are not quite cylindrical but are unevenly shaped; they are provided with varicosities, and between the latter the protoplasm is granular. The main point, however, is the presence of anastomoses between different fibers, resulting in a real framework-like syncytium in the plasma. The difficulties encountered in the fixation of the cultures have made my experiments aiming to a coloration by means of a method supposed to be more or less specific for neuroglia entirely unsuccessful, so aside from the silver-stained preparations I have studied them by means of basic hematoxylin. I cannot, therefore, prove that the fibers just described are neuroglia, but indirect evidence supporting this view can be produced by excluding what we know they are not. Previous investigations (Carrel and Burrows, Ingebrigtsen) have shown that connective tissue and endothelial tissue grow in plasma in a manner quite different from the structures under consideration. As demonstrated from the silver preparations the nerve fibers growing out from ganglia cells also display a different aspect. And then the only conceivable idea concerning their origin is the assumption that these fibers are growing neuroglia tissue.

Fig. 13 A is produced by a combination of three photographs of a culture 3 days old from a piece of cerebellum taken from a young cat. The photographs have been focused on three different planes, following the fibers in their course from their beginning in a big cell of the cortex of cerebellum out to their branches and ends in the plasma. Fig. 13 B is a drawing reproduced from the combined photographs, bringing out details of the preparation which have been omitted in the photographs. These illustrations show how the protoplasmic filaments growing out of the big cell branch, anastomose between themselves, and with other fibers from the neighborhood form a real syncytium built of protoplasmic filaments of different size.

DISCUSSION.

The Wallerian degeneration of a peripheral nerve means the death and disintegration of the axis cylinders and myelin sheath.

From the work of Ranvier[4] we know that the process is influenced in its rate by various agencies; thus it occurs more quickly in young animals than in older ones, in vigorous and healthy individuals than in the sick, more quickly also in warm-blooded than in cold-blooded animals, and in mammals it occurs more quickly in rabbits, for instance, than in dogs.

But in any case the various stages of degeneration follow each other in a typical way quite different from that of a nerve in the dead body.

Mönckeberg and Bethe[5] have observed that the first stages of the Wallerian degeneration may occur in a dead animal when it is kept at body temperature for some hours, but that it stops within the first twenty-four hours. I have confirmed the results of these investigators and found that there is no progress of the degeneration of the nerves after twelve hours in a dead body kept at 38°. As the death of the individual is different from cellular death, the latter occurring later than the former, Mönckeberg and Bethe concluded that the Wallerian degeneration is a process connected with living tissue.

Merzbacher[6] confirming this view added that the occurrence of a Wallerian degeneration in a nerve means that this nerve is in a condition of survival.

Nageotte[1] succeeded in producing the first stages of Wallerian degeneration in nerves kept in survival *in vitro*, and his work has demonstrated that the segmentation of the axis cylinders and myelin sheaths is independent of, and does not, as believed by Ranvier, result from the functions of the protoplasm of the cells of Schwann. The conception of Nageotte concerning the segmentation of the myelin-clad axis cylinders is that the myelin sheath, which is built of living protoplasm rich in mitochondria, digests the axis cylinders in the closed ovoid cavities formed by its segmentation, dying later after having performed this function. This conception of the part played by the myelin sheath in the destruction of the axis cylinder has been adopted by Cajal. The autodigestion of the myelin-clad

[4] Ranvier, L. A., Leçons sur l'histologie du système nerveux, Paris, 1878.

[5] Mönckeberg, G., and Bethe, A., *Arch. f. mikr. Anat.*, 1899, liv, 135.

[6] Merzbacher, *Neurol. Centralbl.*, 1905, xxiv, 150.

axis cylinders develops *in vitro* in a solution containing sodium chloride and salts of some bivalent metal in certain proportions. This observation of Nageotte demonstrates that the segmentation of the myelin sheath obeys the laws of Loeb as does the life of living animals (Nageotte). It obeys also the other laws of biology. It does not occur in nerves kept at 0° nor in those kept at 45° C.

I have found that the segmentation of the myelin sheath is completely abolished in plasma. This does not mean that the nerves kept in plasma are dead; for we know (Carrel and Burrows[7] and Ingebrigtsen[8]) that plasma is by far the best medium for the maintenance of life of tissue outside of the body, and we have a direct proof of the survival of nerves kept in plasma from the fact that in this medium the cells of Schwann give rise to a rich proliferation. And nerve fibers incubated in plasma for twenty-four and forty-eight hours develop a Wallerian degeneration when incubated afterwards in Ringer solution. The proper ratio of calcium and sodium salts is present in plasma as well as in serum, and the addition of calcium chloride did not produce segmentation.

It is difficult to understand how Wallerian degeneration is inhibited in plasma. The reasonable conclusion seems to be that the nerve does not die and degenerate in plasma, because it exists there in extremely favorable conditions, more favorable than those prevailing in the organism, where the peripheral part of a divided nerve must degenerate.

Proliferation of the cells of Schwann does not take place, however, even in plasma unless the first stages of the myelin segmentation have developed in the fibers already before their implantation in the new medium. While fibers removed from the peripheral part of a divided nerve within the first 4 days after section give rise to no growth in plasma, a growth occurred in 16 per cent of the cultures prepared from nerve fibers on the 5th day of Wallerian degeneration, and from later stages an increasing percentage of positive cultures was recorded.

We know that in the peripheral part of a divided nerve in rabbits a hypertrophy of the protoplasm of the cells of Schwann is visible on

[7] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiv, 244.

[8] Ingebrigtsen, R., *Jour. Exper. Med.*, 1912, xvi, 421.

the 4th day; their multiplication begins about the 6th day, when the segmentation of the myelin sheath is well started. From these facts it may be concluded that the cells of Schwann are quiescent and unable to multiply up to a certain stage of myelin and axis cylinder segmentation, and that the latter and not the section of the nerve is the direct cause of their proliferation.

Our interpretation of the protoplasmic structures growing into the plasma from the cut ends of the nerve fibers seems to be conclusive; it is clear from the illustrations that they are direct outgrowths from the cells of Schwann, characterized by a tendency to form a syncytial framework. Is this an indication that such an outgrowth occurs from the cut end of a degenerating nerve (central end of peripheral segment) in the organism, and plays some part in the processes of union and regeneration? I have never seen it and I am not aware that it has been noticed by other observers. But it is probable that such a growth from the cells of Schwann of the peripheral part of a divided nerve extends into the scar tissue forming part of it. That structures of this kind have not yet been detected in the organism does not prove their non-existence. They stain faintly, and curving and bending through the scar tissue it may be difficult to bring out their true form.

The centrifugal orientation of the young axis cylinders growing out from the central part of a divided nerve has been explained by Forssman,[9] and his explanation has been accepted and modified by Cajal[10] on the assumption of positive chemotactic influences exerted by the cells of Schwann of the peripheral part. This assumption is merely hypothetical. An anatomical conception of the centrifugal orientation based upon my experiments seems more satisfactory, assuming that the protoplasmic syncytium of Schwann growing out from the peripheral part of a divided nerve, branching in the scar may receive the axis cylinders coming from the central part and serve as a guidance for them into the peripheral segment. In no case did I observe axis cylinders forming inside or growing out from the incubated nerve fibers. The fact that axis cylinders grow out into the plasma from pieces of the central nervous system containing ganglia

[9] Forssman, J., *Beitr. z. path. Anat. u. z. allg. Path.*, 1898, xxiv, 56; 1900, xxvii, 407.

[10] Cajal, Studien über Nerveregeneration, Leipzig, 1908.

cells, is an additional argument in favor of the monogenetic theory of the regeneration of nerves.

From a morphological standpoint the study of the grown out syncytium of Schwann in the plasma presents some features of interest. The study of the structure of the protoplasm of Schwann is a difficult task, and it is only in the last few years that the work of Nageotte has elucidated its syncytial nature. The protoplasm growing out from the cells of Schwann in the plasma in the first stages of the Wallerian degeneration shows a tendency to form a syncytial network. In the protoplasmic structures growing out in the later stages of Wallerian degeneration the liability to branch and anastomose is not so pronounced; the straight parallel rows of cells are similar to the so called bands of von Büngner.[11] The bands of von Büngner have been interpreted by this investigator and several others after him as the first signs of a differentiation of an axis cylinder out of the multiplied and coalesced cells of Schwann in a degenerating nerve. The errors of this interpretation, however, have been shown by Cajal[10] and Nageotte.[12] My photographs of the syncytium of Schwann growing in plasma, and showing the morphological likeness between the structures and axis cylinders, give an additional demonstration of the error. It is interesting to note the corresponding appearance of a syncytium of Schwann and syncytial neuroglia growing in plasma. There is very little difference between the pictures, and this is not surprising, when we take into consideration the common origin of these tissues in the early fetal life of the organism.

CONCLUSIONS.

1. The Wallerian degeneration occurring in peripheral nerves by incubation in Ringer solution and serum does not occur in plasma.

2. Peripheral nerves incubated in plasma give rise to no growth. The same is true of peripheral nerves in a Wallerian degeneration up to the 4th day.

3. Peripheral nerves in Wallerian degeneration from the 5th day give rise to a growth of the syncytium of Schwann. In cultures from

[11] von Büngner, O., *Beitr. z. path. Anat. u. z. allg. Path.*, 1891, x, 321.

[12] Nageotte, *Compt. rend. Soc. de biol.*, 1911, lxx, 861.

later stages there is a progressive growth of the same structure. It is evident that the proliferation of the cells of Schwann is directly produced by the degeneration of the axis cylinder and its myelin sheath.

4. In no case was growth of axis cylinders observed.

5. The growth of the syncytium of Schwann from degenerating nerves affords a basis for an anatomical conception of the centrifugal orientation of growing axis cylinders in regeneration.

6. Morphologically there is a striking resemblance between the syncytium of Schwann and neuroglia growing in plasma.

EXPLANATION OF PLATES.[13]

Plate 34.

Figs. 1 and 2. Microphotographs of nerve fibers from the sciatic nerve of a rabbit, incubated in plasma for 12 days. Hardened in formalin and stained with hematoxylin. Fig. 1, low power magnification; Fig. 2, high power magnification. There is no trace of Wallerian degeneration.

Fig. 3. Microphotograph of protoplasmic filaments (syncytium of Schwann) emerging into the plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 5 days after section. Hardened after 6 days of incubation and stained with hematoxylin.

In the center of the picture a fiber is seen including a nucleus of Schwann and three myelin (fatty) globules. Several connective tissue cells are also seen.

Plate 35.

Fig. 4. Camera lucida drawing of framework-like syncytium grown out from the cut end of a nerve segment removed from the peripheral part of a divided nerve, 5 days after section and incubated in plasma for 6 days. Stained with hematoxylin.

The nuclei of Schwann are surrounded by myelin globules.

Plate 36.

Fig. 5. Camera lucida drawing of branching protoplasmic filaments grown in plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 6 days after section. Incubated for 5 days and stained with hematoxylin.

---

[13] Dr. O. Berner, of the Anatomical Institute, Christiania, kindly made some of the microphotographs, Dr. Platou a few of the photographs.

PLATE 37.

FIG. 6. Microphotograph of protoplasmic structures grown in plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 6 days after section. Incubated for 9 days. Cajal silver impregnation. Low power magnification.

FIG. 7. Camera lucida drawing from the same preparation. The length of the fibers is about 350 microns.

Note the varicosities produced by nuclei.

PLATE 38.

FIGS. 8 AND 9. Microphotographs of protoplasmic structures grown in plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 13 days after section. Incubated for 5 days and stained with hematoxylin. Fig. 8, low power magnification; Fig. 9, high power magnification.

PLATE 39.

FIGS. 10 AND 11. Microphotographs of protoplasmic structures grown in plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 19 days after section. Incubated for 6 days and stained with hematoxylin. Fig. 10, low power magnification, showing the whole bunch of filaments; Fig. 11, high power magnification, giving details from the same preparation.

PLATE 40.

FIG. 12. Microphotograph of the branching and anastomosing syncytium grown in plasma, from a piece of cerebellum of a young dog. Culture 2 days old stained with hematoxylin. High power magnification (1,900 diameters).

PLATE 41.

FIG. 13. Microphotographs (A) focused on three different planes of branching and anastomosing syncytium grown out from a big neuroglia cell of a piece of cat cerebellum. Culture 3 days old. Cajal silver impregnation. B is a drawing of the same structures.

265

THE REACTIONS BETWEEN BACTERIA AND ANIMAL TISSUES UNDER CONDITIONS OF ARTIFICIAL CULTIVATION.

II. Bactericidal Action in Tissue Cultures.

By HENRY FIELD SMYTH, M.D.

(*From the Laboratory of Hygiene of the University of Pennsylvania, Philadelphia.*)

(Received for publication, October 14, 1915.)

The author has previously[1] reported investigations on the reactions observed when tissue cultures *in vitro* were inoculated with living pathogenic bacteria, and he desires here to record further observations on the subject. In the former experiments the culture medium used was chicken plasma two parts, with Ringer solution one part, with various chick embryo tissues. In the present experiments a dilution of equal parts of plasma and Ringer solution was employed, thus reducing the proportion of plasma and thus of antibodies or other substances inhibiting bacterial growth. With this change in the strength of the plasma corresponding changes were observed in the bacterial reactions, and the object of this paper is to report these changes, especially as observed with *Bacillus typhosus* and *Bacterium diphtheriticum.*

Bacillus typhosus.

In the former series the organism used was an old laboratory strain of the Wyatt-Johnson type, while in the present series in addition to this strain there were also employed three recently isolated strains from clinical typhoid cases.[2] With the stronger plasma first employed the Wyatt-Johnson bacillus refused to grow or even to survive for any length of time. In the present series with more dilute

[1] Smyth, H. F., *Jour. Exper. Med.*, 1915, xxi, 103; *Centralbl. f. Bacteriol.*, *1te Abt.*, *Orig.*, 1915, lxxvi, 12.

[2] These strains were furnished by Dr. Ira Ayer, Pathologist of the Episcopal Hospital, Philadelphia.

plasma there were many purposely very heavy inoculations and in a number of instances some of the organisms survived and formed colonies. In the first series inoculations from 24 hour agar slants were made into a few cc. of Ringer solution, and 1, 2, or 3 loops from this were inoculated into the diluted plasma or into the amount of fresh Ringer solution to be used for dilution, while in the second series many inoculations were made directly from agar into diluted plasma. In all instances, however, there was evident great reduction in the number of organisms developing, and several tests were made to ascertain the extent of this bacteriolysis by the use of agar plates containing a similar amount of the same inoculated Ringer solution (0.05 to 0.1 cc.) to that used in the cultures. Table I, No. 42, and Table II, No. 44, show some of these results. No. 42 shows a great reduction, uncounted in the first dilution cultures and over 1,300 in the second dilution, no bacteria surviving in either. No. 44 also shows the same results, though here the inoculations were not so heavy. Series of cultures were also made with the other recently isolated strains, designated as A1, A2, and A3, and here also the same marked bacteriolytic action of the plasma was evident though not in as high a degree, some colonies always developing except in the second dilutions. The results with A1 are shown in Table II, No. 45. In the first dilution the colonies on the agar plates were too numerous to count, while the plasma cultures averaged little over 150 in the presence of tissue and showed only 27 colonies without tissue. In the second dilution where there was an average of over 90 colonies on agar plates none developed in the plasma cultures with heart tissue or without tissue, and an average of 5.5 colonies developed in the presence of splenic tissue. In Table I, No. 43, are shown similar results with Type A3. Here the first dilution contained 150,000 colonies on agar, while in plasma alone there were no colonies, in the presence of heart tissue an average of 1.5 colonies, and in the presence of splenic tissue 35 colonies per culture. The second dilution showed an average of 55 colonies on agar while no colonies developed in plasma with or without tissue.

The tables also show similar quantitative tests made for comparison with *Bacillus coli verus*, *Bacillus dysenteriæ* (Harris), and *Bacterium diphtheriticum*.

TABLE I.

Series 7. November 5, 1914. 24 Hour Plasma. 15 Day Chick Embryo Tissue.

Stock inoculations.	1 loop from	24 hour agar slant	in 1 cc. of	Ringer	solution.
Dilution A.	1 " "	stock inoculation	" 1 " "	"	"
" B.	1 " "	Dilution A	" 1 " "	"	"

40s	*Bacterium diphtheriticum* inoculations.	
A	Heart tissue.	No bacterial colonies.
	" "	" " "
	Spleen "	Scattered colonies with halo around tissue.
	Plasma alone.	No colonies.
	Agar plates.	" "
B	Heart tissue.	" "
	" "	" "
	Spleen "	5 colonies just beyond area of tissue growth.
	Plasma alone.	No colonies.
	Agar plates.	" "
41s	*Bacillus coli verus* inoculations.	
A	Heart tissue.	Very numerous scattered colonies.
	" "	" " " "
	Spleen "	" " " " } no halos.
	Plasma alone.	" " " " } no halos.
	Agar plates.	Average over 9,000 colonies.
B	Heart tissue.	3 colonies.
	Spleen "	9 "
	Plasma alone.	1 colony.
	Agar plates.	17 colonies.
42s	*Bacillus typhosus* inoculations, Wyatt-Johnson strain.	
A	Heart tissue.	No colonies.
	" "	" "
	Spleen "	" "
	Plasma alone.	" "
	Agar plates.	Very numerous colonies, not counted.
B	Heart tissue.	No colonies.
	" "	" "
	Spleen "	" "
	Plasma alone.	" "
	Agar plates.	1,362 "
43s	*Bacillus typhosus* inoculations, A3 type.	
A	Heart tissue,	1 colony.
	" "	3 colonies.
	Spleen "	35 " around border of culture.
	Plasma alone.	No colonies.
	Agar plates.	150,000 colonies.
B	Heart tissue.	No colonies.
	" "	" "
	Spleen "	" "
	Plasma alone.	" "
	Agar plates.	55 "

TABLE II.

Series 8. November 9, 1914. 5 Day Plasma. 11 Day Chick Embryo Tissue.

Stock inoculations.	1 loop	from	24 hour agar slant	in	1 cc. of Ringer solution.
Dilution A.	2 loops	"	stock inoculation	"	1 " " " "
" B.	2 "	"	Dilution A	"	1 " " " "

44s	*Bacillus typhosus* inoculations, Wyatt-Johnson strain.	
A	Heart tissue.	No colonies.
	" "	" "
	Plasma alone.	" "
	Agar plates.	Very numerous colonies, not counted.
B	Heart tissue.	No colonies.
	" "	" "
	Spleen "	" "
	" "	" "
	Plasma alone.	" "
	Agar plates.	Average 68.5 colonies.
45s	*Bacillus typhosus* inoculations, A1 type.	
A	Heart tissue.	230 colonies, scattered.
	" "	82 " "
	Plasma alone.	27 "
	Agar plates.	Very numerous colonies, not counted.
B	Heart tissue.	No colonies.
	" "	" "
	Spleen "	3 "
	" "	8 " , no halo.
	Plasma alone.	No "
	Agar plates.	Average 92.5 colonies.
46s	*Bacillus dysenteriæ* (Harris) inoculations.	
A	Heart tissue.	137 colonies, scattered.
	" "	1,000+ " "
	Plasma alone.	200+ "
	Agar plates.	None made.
B	Heart tissue.	1 colony.
	" "	2 colonies.
	Spleen "	1 colony away from tissue.
	" "	6 colonies.
	Plasma alone.	No "
	Agar plates.	Average 157 colonies.

In this series the splenic tissue was too young to have much deleterious action on the organisms, as at 11 days there is very little free cell migration.

Bacillus coli verus.

Bacillus coli verus (Table I, No. 41) shows only a slight reduction; Dilution A was not counted, but there appeared to be as many colonies in plasma as in agar, and Dilution B shows an apparent reduction from an average of 17 colonies on agar to 9 with splenic tissue, 3 with heart tissue, and 1 with plasma alone, all of which might well be due to irregularities in distribution, as such small amounts of inoculated Ringer solution are used in making cultures. These results agree with those previously reported for this organism, though no actual counts were then made.

Bacillus dysenteriæ.

No tests were made with this organism in the former series. In the present series (Table II, No. 46) *Bacillus dysenteriæ* seems to occupy a position between the extremes of *Bacillus typhosus* and *Bacillus coli verus*, there being an evident marked bacteriolysis but decidedly less than with *Bacillus typhosus*. The average of 157 bacilli in Dilution B on agar was reduced in plasma with heart tissue to 1.5 and with splenic tissue to 3.5, while there were no colonies in plasma without tissue. There was also an evident reduction in Dilution A cultures, though no counts were made on agar.

Bacterium diphtheriticum.

In the former series with the stronger plasma three strains of *Bacterium diphtheriticum* were employed, Park-Williams Bacillus 8 and two freshly isolated strains from clinical cases. In the present series with the 50 per cent plasma ten different strains were used, Bacillus 8 and nine recently isolated organisms. In general the results agree with those already reported, though here also the effect of plasma dilution is seen in the survival of many organisms away from the presence of tissue, but even when many such do survive, the colonies are more numerous and larger around the tissue fragments. In the former series with 66 per cent plasma diphtheria colonies developed only closely surrounding tissue fragments, giving to each fragment the appearance of a sun in a miniature solar system. Table I, No. 40, and Tables III, IV, and V show the results of these cul-

tures. In Table IV larger box cultures, with two or more times as much plasma as in the cover-glass cultures designated as s, were used, and in Table V still larger cultures in Petri dishes, with 1 cc. of plasma. This accounts for the much higher bacterial counts in agar plates and lessens the influence of tissue cells as the amount of

TABLE III.

Series 13. January 15, 1915. 1 Day Plasma. 13 Day Chick Embryo Tissue.

Bacterium diphtheriticum inoculations. All cultures from 1st dilution.

82s	Strain 2 (Bacillus 8)	Heart tissue.	5 colonies.
		" "	1 colony.
		Spleen "	1 " not near tissue.
		" "	2 colonies " " "
83s	Strain 3	Heart "	2 " near tissue.
		Spleen "	1 colony not near tissue.
84s	" 4	Heart "	2 colonies.
		Spleen "	1 colony, not near tissue.
		" "	1 " " " "
85s	" 5	Heart "	12 colonies, scattered.
		" "	23 " "
		Spleen "	16 " not near tissue.
		" "	57 " " " "
86s	" 6	Heart "	4 " , scattered.
		" "	2 "
		Spleen "	2 " not near tissue.
87s	" 7	Heart "	5 " , scattered.
		" "	3 " "
		Spleen "	4 " "
		" "	9 " "
88s	" 8	Heart "	3 " "
		Spleen "	3 " "
89s	" 9	Heart tissue.	Many scattered colonies.
		" "	" " "
		Spleen "	" " " avoiding tissue.
		" "	" " " " "
90s	" 10	Heart tissue.	2 colonies.

TABLE IV.

Series 14. January 20, 1915. Fresh Plasma. 14 Day Chick Embryo Tissue.

Bacterium diphtheriticum inoculations. All cultures from 1st dilution.

91B	Strain 2 (Bacillus 8)	Heart tissue.	No colonies.
		" "	1 colony near tissue.
		Spleen "	2 colonies, avoiding tissue.
		" "	No "
		Agar plate.	2 "
92B	Strain 3	Heart tissue.	Numerous colonies, scattered.
		" "	" " clustered about tissue.
		Spleen "	No colonies.
		" "	80 " mostly avoiding tissue.
		Agar plate.	196 "
93B	" 4	Heart tissue.	5 " clustered around tissue.
		" "	10 " " " "
		Spleen "	12 " avoiding tissue.
		" "	8 " " "
		Agar plate.	16 "
94B	" 5	Heart tissue.	14 " clustered around tissue.
		" "	21 " " " "
		Spleen "	No "
		" "	" "
		Agar plate.	56 "
96B	" 7	Heart tissue.	34 " " and scattered.
		" "	44 " " all but 3.
		Spleen "	18 " avoiding tissue.
		" "	4 "
		Agar plate.	140 "
97B	" 8	Heart tissue, 5 day embryo.	65 colonies, scattered.
		" " " " "	Numerous colonies, clustered and scattered.
		Agar plate.	76 colonies.
99B	" 10	Heart tissue " " "	66 " clustered.
		" " " " "	94 " " about tissue and scattered.
		Agar plate.	110 colonies.

In this series larger cultures on glass box lids were used to bring out more clearly the influence of splenic tissue cells.

TABLE V.

Series 15. February 3, 1915. Fresh Plasma. 14 Day Chick Embryo Tissue.

Bacterium diphtheriticum inoculations. All cultures from 1st dilution.

106P	Strain 1	Heart, spleen, and liver tissue. " " " " " Agar plate.	No colonies. " " 352 "
107P	" 3	Heart and liver tissue. " " " " Agar plate.	No " Many colonies, scattered and clustered. 1,300 colonies.
108P	" 4	Heart, spleen, and liver tissue. " " " " " Agar plate.	Many " , clustered and scattered. Many colonies, clustered and scattered. 5,000 colonies.
109P	" 5	Heart tissue. " " Agar plate.	2 colonies near tissue. 26 " clustered and scattered. 140 colonies.
110P	" 6	Heart tissue. " " Agar plate.	1 colony near tissue. No colonies. 700 "
111P	" 7	Heart tissue. " " Agar plate.	No " " " 1,000 "
112P	" 8	Heart tissue. " " Agar plate.	Many colonies, clustered and scattered. Many colonies, scattered. 3,000 "
113P	" 10	Heart tissue. " " Agar plate.	50 " clustered. 179 " " 5,000 "

tissue per culture was not proportionably increased. Table I, No. 40, shows almost complete destruction of organisms in the presence of splenic tissue and plasma, and complete destruction with heart tissue and with plasma without tissue. However, here the cultures

must have contained very few organisms, as none developed when transfers were made to agar plates. In the small cultures of Table III the influence of dilution is seen in the scattering of the colonies, the organisms in some cases being able to overcome the influence of plasma even without the aid of tissue, but the larger cultures of Tables IV and V show more characteristic groupings and a marked destruction of bacteria, as seen by comparison of the plasma cultures with the agar plate counts. This destruction becomes more marked the larger the cultures, as is seen in Table V. All show the bactericidal action evident in the immediate vicinity of splenic fragments with the characteristic clear bacteria-free halos around the tissue fragments. This action is pronounced with *Micrococcus aureus* cultures, as previously noted, and is also seen at times with *Bacillus typhosus*, as in Table I, No. 43 A.

Influence of Tissue on Bacteria.

In addition to the bactericidal influence of growing splenic tissue noted above, which seems to be nearly coextensive with the area of migration of white cells in the plasma, and which must be due to such cells or their products, Tables I and II show another interesting action of tissue. These tables show that even where this bacteria-free halo is evident, often with *Bacillus typhosus*, at times with *Bacterium diphtheriticum* and with *Bacillus dysenteriæ*, a greater number of organisms may survive in the presence of splenic tissue than in heart cultures, and almost always more in the presence of any tissue than in plasma without tissue. This seems to show that the bacteria are either encouraged by the tissue or tissue products directly or are protected by them from the lytic action of the plasma. The spleen thus seems to have a double action, antagonistic in the immediate area of white cell migration and favorable in the area just beyond, probably due to cell secretions or excretions diffusing out from the tissue. This same double action of splenic cultures will be brought out in connection with tubercle bacillus cultures in a subsequent paper.

SUMMARY.

The results here reported confirm those of the former papers and strengthen the conclusions drawn therefrom. They may be summarized as follows:

Chicken plasma has a marked bactericidal action on *Bacillus typhosus*, which may be in some slight degree overcome by the presence of growing tissue, especially splenic tissue, in the cultures.

On *Bacillus dysenteriæ* this bactericidal action of chicken plasma is present, but much less marked, and the same counteracting action of tissue, especially splenic tissue, is evident.

On *Bacillus coli verus* chicken plasma has little or no bactericidal action.

On *Bacterium diphtheriticum* chicken plasma has a very strong bactericidal action which may be strongly counteracted by the presence of growing tissue in the cultures.

In all cases the bactericidal action of the plasma is decidedly diminished by dilution, as shown by the comparative results of these and the cultures formerly reported.

The migrating white cells from splenic cultures, or substances closely associated with these cells, have a distinctly bactericidal influence on all organisms tested except *Bacillus coli verus*. Murphy[3] states that lymphocytes first appear in the general circulation of the chick embryo on the 18th to 20th day, but in my cultures of splenic tissue cells resembling lymphocytes in morphology and behavior begin to appear in cultures of 11 or 12 day spleen and are abundant in cultures of spleen from 14 day or older embryos.

[3] Murphy, Jas. B., *Jour. Exper. Med.*, 1914, xix, 181

THE REACTIONS BETWEEN BACTERIA AND ANIMAL TISSUES UNDER CONDITIONS OF ARTIFICIAL CULTIVATION.

III. THE ACTION OF BACTERIAL VACCINES ON TISSUE CULTURES IN VITRO.

By HENRY FIELD SMYTH, M.D.

(*From the Laboratory of Hygiene of the University of Pennsylvania, Philadelphia.*)

(Received for publication, October 14, 1915.)

While studying the action of pathogenic bacteria on chick embryo tissues cultivated *in vitro* by the method of Burrows,[1] the author was impressed by the apparent stimulation of the cultures inoculated with *Bacillus typhosus* when the bacilli themselves failed to develop. To determine if this was an actual stimulation of growth or merely an accidental condition in the few cultures so far tested, heavy vaccines, or suspensions of killed organisms, were prepared and used for diluting the plasma for tissue cultures, thus allowing the use of many more bacilli per tissue culture than could be used with living organisms. In moderate numbers, up to many thousands per drop, typhoid bacilli will not develop in chicken plasma cultures,[2] but by increasing the number of bacilli to the hundred thousands a few will develop in 50 per cent plasma. In the vaccine cultures, however, suspensions containing one billion or more bacilli per cc. were employed.

The author has previously reported[3] some preliminary results with the use of vaccines of *Bacillus typhosus* as well as of several other organisms with tissue cultures, but at that time no definite measurements were made for comparison with normal controls. Since then a number of other cultures have been made, and in each series estimates as accurate as possible have been made of the extent of new

[1] Smyth, H. F., *Jour. Exper. Med.*, 1915, xxi, 103.

[2] Smyth, H. F., *Jour. Exper. Med.*, 1916, xxiii, 265.

[3] Smyth, H. F., *Centralbl. f. Bacteriol.*, *1te Abt.*, *Orig.*, 1915, lxxvi, 12.

growth in normal controls with plasma diluted with sterile Ringer solution and of cultures in plasma with equal amounts of the various vaccines suspended in Ringer solution. The latter results in general confirm those previously reported, except in the case of *Bacterium diphtheriticum* which in the preliminary tests seemed to have no definite action on tissue growth, but in the later more accurate tests proved to be somewhat stimulating to tissue cell growth although not so much so as were either *Bacillus typhosus* or *Bacillus coli verus*.

In estimating the extent of new growth in some cases three factors were considered, as in Tables I and II, and in others only two, as in Table III. The first figure given represents the mean diameter of the culture expressed in terms of that of the original fragment, the second figure is determined from the scale on the fine adjustment of the microscope, each unit representing ten points in the distance between the clearest focal points of the top and bottom of the cultures, while the third figure is an arbitrary one based on the observer's judgment as to the compactness of the new cells at the mid-focal point. From the results of many comparative measurements it would seem that as accurate determinations can be made by omitting the measurement of depth and considering only the extent of growth and density of cells as was done in Table III. These tables are given to show the method by which the results here reported were obtained, and they represent only a few of the cultures made. Table I shows results with vaccines of *Bacillus typhosus* and *Micrococcus aureus*, the temperatures at which the vaccines were prepared being stated in each case. All vaccines were from 24 hour agar slants. In Table II *Micrococcus aureus* and two strains of *Bacillus typhosus* were employed: W-J an old laboratory strain of Wyatt-Johnson, and A1 a recently isolated strain from a clinical typhoid case. In this table vaccines killed at low temperatures were compared with others killed at 100° C. In Table IV, in addition to the organisms employed in Tables I and III, vaccines of *Bacterium diphtheriticum* were used. As these were from fresh agar cultures they were of course free from toxin.

Table IV gives a summary of all the results of these series, except a few cultures of nervous tissue (cerebral vesicles and cord) which

TABLE I.

Series 5. October 29, 1914. Fresh Plasma. 12 Day Chick Embryo Tissue. Measurements Taken after 2 Days.

Plasma + 56° C. vaccine, *B. typhosus*, Strain W-J.

		diameter depth density	*total volume*	
28s	Heart tissue.	12 x 2.5 x 3	−90	
	" "	9 x 3 x 2	−54	Average 66
	" "	6 x 3 x 3	−54	
	Spleen "	8 x 2.5 x 3	−60	
	" "	9 x 2 x 2	−36	" 40
	" "	6 x 2 x 2	−24	

Plasma + 60° C. vaccine, *B. typhosus*, Strain W-J.

29s	Heart tissue.	7 x 2.5 x 2	−35	
	" "	5.5 x 3.5 x 2	−38.5	Average 36.75
	Spleen "	6 x 2.5 x 2	−30	
	" "	8 x 2.5 x 2	−40	" 33.3
	" "	5 x 3 x 2	−30	

Plasma + 60° C. vaccine, *M. aureus*.

30s	Heart tissue.	3.5 x 1 x 2	− 7	
	" "	7 x 2 x 2	−28	Average 17.6
	" "	4.5 x 2 x 2	−18	
	Spleen "	5 x 1.5 x 2	−15	
	" "	6 x 2.5 x 3	−45	" 35
	" "	6 x 2.5 x 3	−45	

Plasma + sterile Ringer solution.

31s	Heart tissue.	6 x 3 x 2	−36	
	" "	6 x 2 x 2	−24	Average 23
	" "	3 x 1.5 x 2	− 9	
	Spleen "	6 x 2 x 2	−24	" 24
	" "	6 x 2 x 2	−24	

Aureus vaccine depresses heart tissue, but stimulates spleen tissue.
Typhosus vaccine stimulates " " and " " "

TABLE II.

Series 6. November 4, 1914. Fresh Plasma. 14 Day Chick Embryo Tissue. Measurements Taken after 2 Days. Vaccines All Standardized 1,000,000,000 Bacteria per Cc.

Plasma + sterile Ringer solution.						
		diameter	*depth*	*density*	*total volume*	
32s	Spleen tissue.	3	x 2	x 3	−18	Average 21
	" "	6	x 2	x 2	−24	
Plasma + 56° C. vaccine, *M. aureus*.						
33s	Spleen tissue.	5	x 2	x 2	−20	
	" "	5.5	x 2	x 2	−22	Average 22
	" "	4	x 2	x 3	−24	
Plasma + 100° C. vaccine, *M. aureus*.						
34s	Spleen tissue.	3.5	x 2	x 3	−21	
	" "	3	x 2	x 3	−18	Average 17
	" "	4	x 1.5	x 2	−12	
Plasma + 56° C. vaccine, *B. typhosus*, Strain W-J.						
36s	Spleen tissue.	7	x 2.5	x 2	−35	
	" "	5	x 2	x 3	−30	Average 31.6
	" "	5	x 2	x 3	−30	
Plasma + 100° C. vaccine, *B. typhosus*, Strain W-J.						
37s	Spleen tissue.	7	x 2.5	x 2	−35	Average 30
	" "	5	x 2.5	x 2	−25	
Plasma + 56° C. vaccine, *B. typhosus*, Strain A1.						
38s	Spleen tissue.	6	x 2.5	x 3	−45	
	" "	4	x 3	x 3	−36	Average 33
	" "	3	x 2	x 3	−18	
Plasma + 100° C. vaccine, *B. typhosus*, Strain A1.						
39s	Spleen tissue.	4	x 2	x 4	−32	
	" "	7	x 2.5	x 3	−52.5	Average 40.16
	" "	8	x 1.5	x 3	−36	

Aureus vaccine at 100° C. depresses spleen tissue.
" " " 56° C. slightly stimulates spleen tissue.
Typhosus " " 100° C. stimulates spleen tissue.
" " " 56° C. " " "

TABLE III.

Series 12. January 10, 1915. Fresh Plasma. 12 Day Chick Embryo Tissue. Measurements Taken after 4 Days; No Account Taken of Depth.

		Plasma + sterile Ringer solution.				
		diameter		*density*	*total volume*	
75s	Heart tissue.	2.5	x	2	−5	
	" "	3.5	x	1	−3.5	Average 5.2
	" "	3.5	x	2	−7	
	Spleen "	3	x	2	−6	
	" "	3	x	2	−6	" 5.3
	" "	2	x	2	−4	
		Plasma + 100° C. vaccine, *B. typhosus*, Strain W-J.				
77s	Heart tissue.	5	x	2	−10	
	" "	8	x	2	−16	Average 13
	Spleen "	3	x	2	− 6	
	" "	4	x	2	− 8	" 8
	" "	5	x	2	−10	
		Plasma + 60° C. vaccine, *M. aureus*.				
78s	Heart tissue.	1.25	x	1	−1.25	
	" "	2.5	x	3	−7.5	Average 4.37
	Spleen "	1.5	x	3	−4.5	
	" "	2	x	2	−4	" 4.25
		Plasma + 100° C. vaccine, *M. aureus*.				
79s	Heart tissue.	4	x	2	−8	
	" "	1.25	x	2	−2.5	Average 5.25
	Spleen "	2.5	x	3	−7.5	
	" "	3	x	2	−6	" 6.5
	" "	3	x	2	−6	
		Plasma + 100° C. vaccine, *B. coli verus*.				
80s	Heart tissue.	5	x	2	−10	
	" "	6	x	2	−12	Average 9.3
	" "	3	x	2	− 6	
		Plasma + 1,000° C. vaccine, *B. coli verus*.				
80s	Spleen tissue.	4	x	2	−8	
	" "	3	x	2	−6	Average 7.3
	" "	4	x	2	−8	

Typhosus vaccine stimulates heart and spleen tissue.
Aureus " at 60° C. depresses heart and spleen tissue.
" " at 100° C. does not depress heart, and stimulates spleen tissue.
Coli verus " at 100° C. stimulates heart and spleen tissue.
The totals are small in this series as only two measurements were considered.

showed no definite results. Accurate comparative determinations of the amount of growth would be difficult to make, however, with these tissues as their growth is very irregular and varied in character under perfectly normal conditions. The vaccines tabulated as old were kept in sealed glass tubes at room temperature for over 6 months. The results with these old vaccines should not be given too much weight, as only one series of cultures was made with each, except in the case of *Micrococcus aureus* where two series were made of each. In this case it will be noted that whereas with the fresh 60° vaccines splenic tissue seems to be somewhat inhibited, it seems to be stimulated when the vaccine is heated to 100° C. or is kept for some months. The action of *Micrococcus aureus* vaccine on growth from heart tissue is variable, but never so actively stimulating as that of the typhoid or colon organisms, and at times is decidedly inhibitory. This inhibitory action when present is more pronounced than would appear from this table. The results of Table IV may be summarized as follows: *Bacillus typhosus* vaccines stimulate cell growth under all conditions. *Bacillus coli verus* vaccines when freshly prepared actively stimulate cell growth. *Bacterium diphtheriticum* vaccines mildly stimulate cell growth under all conditions. *Micrococcus aureus* vaccines seldom stimulate and usually inhibit cell growth when freshly prepared. Old vaccines lose this inhibitory action and seem to stimulate growth.

SUMMARY AND CONCLUSIONS.

These results seem to indicate that many bacteria may be utilized by tissue cells as food for growth or may contain a substance or substances stimulating cell growth or multiplication. This substance is stable and is not destroyed by heating to 100° C. or by long standing.

With *Micrococcus aureus* this action is often neutralized or overcome by a substance inhibitory to growth.

The nature of these substances has not yet been determined, though several attempts along this line were made by endeavoring to split the typhoid bacterial substance according to the method of Vaughan[4]

[4] Vaughan, V. C., Vaughan, V. C., Jr., and Vaughan, J. W., Protein Split Products in Relation to Immunity and Disease, Philadelphia and New York, 1913.

TABLE IV.

Summary of Vaccine Cultures with Tissue.

		Heart tissue.	Spleen tissue.
B. typhosus.	56°	6 series, 14 cultures +	4 series, 12 cultures +
	60°	4 " 10 " +	5 " 13 " +
		1 " 2 " 0	
		1 " 6 " —	
	100°	4 " 12 " +	5 " 15 " +
		1 " 7 " —	
" " old.	100°	1 " 3 " —	1 " 3 " +
B. coli verus.	60°	1 " 3 " +	1 " 3 " — (all old).
	100°	2 " 7 " +	2 " 5 " +
" " " old.	10°	1 " 2 " +	1 " 3 " —
M. aureus.	56°	1 " 3 " +	1 " 3 " +
	60°	5 " 17 " —	2 " 5 " —
			2 " 6 " +
	100°	2 " 5 " +	2 " 5 " +
		1 " 2 " 0	
		1 " 3 " —	2 " 5 " —
" " old.	60°	2 " 6 " +	2 " 6 " +
	100°	1 " 3 " +	2 " 6 " +
		1 " 3 " —	
Bacterium diphtheriticum.	60°	3 " 6 " +	3 " 9 " +
" " old.	60°	1 " 3 " —	1 " 3 " +
	100°	1 " 3 " +	1 " 3 " +

+ = stimulation; 0 = no action; — = depression.

and testing the poisonous and non-poisonous residues separately. However, at the time too little bacterial substance was used to obtain enough end-products to be of much use, and the products so obtained prevented the plasma from coagulating. Even in the uncoagulated plasma there appeared to be an increase of cells in the cultures with the non-poisonous residue. The author hopes to be able to repeat these tests with split products at a later time when more bacterial substance is available, with the hope of obtaining more definite results.

THE REACTIONS BETWEEN BACTERIA AND ANIMAL TISSUES UNDER CONDITIONS OF ARTIFICIAL CULTIVATION.

IV. The Cultivation of Tubercle Bacilli with Animal Tissues in Vitro.

By HENRY FIELD SMYTH, M.D.

(*From the Laboratory of Hygiene of the University of Pennsylvania, Philadelphia.*)

Plates 42 to 47.

(Received for publication, October 14, 1915.)

The results obtained from the study of the reaction between various pathogenic bacteria and animal cells under conditions of artificial cultivation according to the method of Burrows,[1] led the author to undertake during the early part of 1915 the study of the action of human tubercle bacilli with chick embryo tissues cultivated in chicken plasma. Two strains of bacilli were employed in this work, one freshly isolated by the author from human sputum, using Petroff's egg-beef-juice-gentian-violet medium, and one obtained from the laboratories of the H. K. Mulford Co., a "Baldwin" bacillus used by them for preparing tuberculin.

Technique.

Inoculations were made from Petroff slants or from glycerine agar into sterile Ringer solution, and the inoculated saline was used for diluting the plasma (equal parts of plasma and saline) for cultures. In most cases the inoculated saline was simply shaken vigorously for a few moments to distribute the bacillary masses, but in a few instances it was shaken with sterile glass beads and then filtered through sterile glass-wool to remove all clumps. In each series a

[1] Smyth, H. F., *Jour. Exper. Med.*, 1915, xxi, 103; *Centralbl. f. Bacteriol.*, 1te *Abt., Orig.*, 1915, lxxvi, 12.

number of cultures of the same tissues were made with and without bacilli, and specimens were fixed and stained at varying intervals after starting incubation.

Cultures were made on cover-glasses sealed on deep celled culture slides and also on the covers of glass boxes from one to two inches in diameter. All were sealed with soft paraffin and incubated at 38° C. Cultures were fixed in absolute alcohol, and when containing bacilli were stained for one or more hours with cold carbol-fuchsin, decolorized with acidified alcohol, and counterstained with Giemsa stain. This gave deep blue nuclei, faintly stained cell protoplasm, pink plasma, and clear bright red bacilli.

Colony Formation.

In these plasma cultures with or without tissue the tubercle bacilli develop freely with the formation of characteristic colonies. In specimens stained after three or more days there appear small, closely woven, filamentous colonies and frequently larger masses of irregularly twisted, loose, parallel threads forming an open network and resembling a tangled skein of spun silk. Where colonies encroach on tissue fragments these tangled skeins may be seen separating islands of tissue cells. In these colonies single organisms are hard to distinguish, being mostly arranged as solid or beaded threads resembling ordinary streptothrix colonies. Examples of this arrangement are shown in Figs. 1, 2, and 7. Fig. 1 also shows a compact colony, both colonies being shown under too low magnification to bring out single bacilli. Fig. 7 A, showing under the oil immersion lens a very small beginning colony, brings out the fact that the threads in these colonies stain irregularly, as do also streptothrix threads. This colony formation in loose skeins gives the picture described by Koch[2] but seldom seen in the solid media in general use at present.

Phagocytosis of Bacilli.

Phagocytosis of tubercle bacilli was clearly demonstrated in many cultures of splenic tissues and heart cultures (connective tissue cells)

[2] Koch, R., *Mitt. a.d. k. Gsndhtsamte.*, 1884, ii, 1.

as well as cells from skin and liver fragments. Fig. 2 shows new cells from heart tissue loaded with bacilli, Fig. 3 new cells from a fragment of epithelium from a four day embryo with a few bacilli, and Fig. 4 shows an epithelial plate from a liver fragment with a cluster of phagocytyzed bacilli. In all these cases careful focussing showed that the bacilli were within and not on the cells and they usually had a tendency to avoid the nucleus. In epithelial and connective tissue cultures the bacilli seem to have no especial attraction for the new cells, but are merely acted upon when the cells happen to come in contact with them in their wanderings, as all embryonal cells of whatever type are more or less actively motile in tissue cultures, or at least have motile processes.

Splenic Tissue Cultures.

The most interesting results, as would naturally be expected, were obtained from cultures of splenic tissue from fourteen day or older embryos. One series from a twelve day embryo, at which time there are few if any lymphocytes formed, gave the same picture as with heart tissue, occasional phagocytosis by connective tissue cells but no cell attraction. The most characteristic results were obtained with fourteen day splenic tissue, at which time, in cultures *in vitro* at least, lymphocytes or lymphocyte-like cells are very abundant and migrate freely over the entire field.[3] When a fairly heavy inoculation of unfiltered organisms with many small clumps is employed, the small round cells from the splenic tissue have a tendency to cluster around the clumps of bacilli as shown in Figs. 5 to 8. This clustering of lymphatic elements was observed as early as the third day with fourteen day tissue (Fig. 5) and within twenty-four hours in a culture from eighteen day spleen (see Fig. 8 B where small cells are seen on the surface of a large clump of bacilli). In Fig. 7 B is seen a loose cluster of bacilli and cells, in this case found floating in the serum from the culture in which were seen the other forms in the same sketch.

In all the series of cultures made by the author with tubercle bacilli the tendency of the new cells and migrating cells from splenic tissue

[3] Smyth, H. F., *Jour. Exper. Med.*, 1916, xxiii, 265.

to group in clumps or clusters throughout the plasma rather than to be evenly distributed was much more marked than in the normal bacteria-free controls, which of course were made with each series.

Giant Cell Formation.

Following the clustering of the lymphocytes around the bacilli the larger parenchymatous cells from the spleen, those of epithelioid type, approach, as shown in Fig. 5, and phagocytyze all bacilli with which they come in contact, seeming actively to seek out bacilli in a way the connective tissue and other cells do not. This is well seen in Fig. 6 B. These epithelioid cells containing bacilli, many of them with two or more nuclei, as is common in cultures *in vitro*, tend to fuse when they come in contact with each other as seen in Figs. 6 C, 8 A, and 9. In Fig. 9 A is a single mononuclear cell; B, B are cells with two nuclei and containing bacilli; C shows beginning fusion of three mononuclears; D of a binuclear with trinuclear cell; and E represents a completely fused multinuclear cell with bacilli. This process of fusion continues until by the seventh day many fully developed giant cells with numerous bladder-like nuclei arranged in characteristic groups, crescentic, bipolar, horseshoe-like, or circular, are seen. After this time these giant cells predominate, the smaller single cells gradually falling out of the plasma as it becomes liquefied by the cell ferments. Fig. 10 shows under a one inch objective an entire fragment of splenic tissue with degenerating reticulated center, radiating connective tissue growth, and many free giant cells scattered through the plasma. Figs. 11 and 12 are fully formed giant cells from a nine day culture, showing in Fig. 11 a cell with rounded margins embedded in deep plasma, and in Fig. 12 a surface cell spread out between plasma and cover-glass and having characteristic indistinct repand margins. The protoplasm in the center of these cells, particularly around the bacilli, appears degenerated and granular or structureless, while that around the nuclear groups is reticulate and healthy. Fig. 12 also shows in the small, dark blue masses the nuclear fragments of included lymphocytes.

In cultures up to three days many mitotic figures may be observed in the larger cells, but after the fusion of cells is fairly under way no

more figures are seen and they never appear in the fully formed giant cells. This supports the theory that tubercular giant cells are fusion cells, like Lambert's foreign body giant cells[4] and not due to the rapid division of nuclei without cell division. Evans, Bowman, and Winternitz[5] as the result of experiments with vitally stained rabbits, describe giant cells formed from epithelioid cells of the liver capillaries by nuclear division without cell division, but from their pictures the author believes that they correspond with the polynuclear cells seen in one to three day cultures as precursors of fully formed fusion giant cells.

Figs. 6 D and 7 C show what the author terms shadow cells, often seen in old cultures and interpreted as cells which having phagocytyzed bacilli are not able to digest them but are exhausted by the bacilli which grow in the cell, causing it to undergo degeneration until nothing but an indistinct shapeless mass remains. All stages of this process may be seen in cultures a week or more old.

Inoculations of Suspensions Free from Clumps.

The conditions noted above are those seen in cultures with heavy inoculations containing clumps of bacilli. Where the bacillary suspensions are first filtered to remove clumps these changes do not appear. In one series with a small 1 mm. loopful of turbid filtrate per 2 cc. of diluted plasma, and in another with three large 4 mm. loopfuls per 4 cc. of diluted plasma no bacilli developed in the neighborhood of either heart or spleen fragments, though a few small irregular acid-fast clumps were occasionally seen. However, these may have been artefacts due to deposits in the carbol-fuchsin. In another series made with a filtered culture and with a somewhat heavier inoculation, splenic cultures showed an occasional small loose cluster of bacilli with lymphocytes in cultures stained up to three days. In older cultures up to nine days no bacilli were observed within the areas of cell growth except at or near the limits of cell migration. In ten and eleven day cultures where the cells were degenerating, a few more bacilli were seen with one or two skein colonies, but still

[4] Lambert, R. A., *Jour. Exper. Med.*, 1912, xv, 510.

[5] Evans, H. M., Bowman, F. B., and Winternitz, M. C., *Jour. Exper. Med.*, 1914, xix, 283.

no bacilli near the tissue fragments or where new cells were plentiful. In a twelve day heart tissue culture from the same series the entire plasma was loaded with colonies and loose bacilli both near and away from tissue cells.

In another series using a bacillary suspension of the Baldwin type the suspension was shaken very vigorously with sterile glass beads before inoculation but was not filtered, and two and six drops respectively were added to 3 cc. of diluted plasma. Clusters of bacilli with lymphocytes were seen in twenty-four hour cultures, as well as epithelioid cells with phagocytyzed bacilli and a few large clumps of bacilli with lymphocytes adherent, as in Fig. 8 B. In eight day cultures a few giant cells were present, but not as many as with the freshly isolated strain of bacilli.

In cultures where the bacilli developed freely the splenic cultures contained more organisms than did those of other tissues, but in filtered cultures and light inoculations splenic cultures showed decidedly fewer organisms than did other tissue cultures. This agrees with results reported by the author with other organisms[3] where splenic tissue appeared to have a double influence: antagonistic, due to the action of the white blood cells or their products; and favorable, due to some other unrecognized cell substance or secretion.

The difference in action between cultures containing clumps of bacilli and those containing separated organisms only is one of mass action and corresponds to similar differences noted by Ayer[6] in his experiments on rabbits where unfiltered suspensions sprayed into the bronchi caused cavity formation while filtered suspensions did not.

SUMMARY.

In plasma cultures, with or without tissue, tubercle bacilli form characteristic streptothrix-like colonies of loosely twisted skeins of threads.

In plasma tissue cultures embryonal connective tissue and epithelial cells phagocytyze tubercle bacilli freely.

Splenic tissue cultures from fourteen day or older embryos if inoculated with isolated bacilli will phagocytyze and prevent the develop-

[6] Ayer, I., *Jour. Med. Research*, 1914, xxx, 141.

ment of all or nearly all bacilli present. If heavy inoculations of tubercle bacilli with many bacillary clumps are made in splenic cultures the bacilli develop more freely than in similar connective tissue cultures from heart tissue in spite of marked phagocytosis. In such cultures the first change seen is a clustering of small round cells, interpreted as lymphocytes, around bacillary clumps and colonies followed by a similar clustering of larger parenchymatous polygonal cells with one or several nuclei, interpreted as epithelioid cells, which phagocytyze any bacilli with which they may come in contact. The larger cells containing bacilli then fuse to form larger multinuclear cells and these by further fusion form typical giant cells with quiescent nuclei in characteristic groupings and bacilli in degenerated protoplasm away from the nuclear groups.

Cells which have phagocytyzed bacilli may digest them or may be unable to do so, in which case the bacilli develop within the cells and eventually cause complete cell degeneration.

CONCLUSIONS.

In plasma tissue cultures *in vitro* with tissue containing lymphatic elements the changes characteristic of early tubercle formation may be seen when such cultures contain masses of tubercle bacilli: First, clustering of lymphocytes about bacilli; second, clustering of epithelioid cells and other polynuclear or polymorphonuclear cells with multiplication of nuclei in the epithelioid cells; third, fusion of epithelioid cells to form giant cells; and fourth, degeneration of cells containing bacilli.

EXPLANATION OF PLATES.

Plate 42.

Fig. 1. Tubercle bacillus colonies from 11 day culture from 14 day chick embryo spleen in chicken plasma inoculated with human tubercle bacilli, showing compact and loose skein colony. No tissue is shown. Drawn from Leitz oc. 4, obj. 6.

Fig. 2. Cells from 7 day culture of 10 day chick embryo heart tissue in chicken plasma inoculated with human tubercle bacilli, showing loose skein colony and new cells with phagocytyzed bacilli. Drawn from oil immersion lens $\frac{1}{12}$.

PLATE 43.

FIG. 3. New cells from 2 day culture of skin from 4 day chick embryo in chicken plasma inoculated with human tubercle bacilli, showing phagocytyzed bacilli. Drawn from $\frac{1}{12}$ oil immersion lens.

FIG. 4. Epithelial plate from 3 day culture of liver tissue from 7 day chick embryo in chicken plasma inoculated with human tubercle bacilli, showing phagocytyzed bacilli. Drawn from Leitz oc. 4, obj. 6.

PLATE 44.

FIG. 5. Cell clusters from 3 day culture of splenic tissue from 14 day chick embryo in plasma inoculated with human tubercle bacilli, showing clustering of small round cells around clumps of bacilli with a few larger parenchyma cells approaching. Drawn from Leitz oc. 4, obj. 6.

FIG. 6. Cells from 7 day culture of splenic tissue from 14 day chick embryo in plasma inoculated with human tubercle bacilli. A represents a loose cluster of tubercle bacilli and small round cells; B, a parenchyma cell with several nuclei approaching and phagocytyzing bacilli; C, a clump of fused parenchyma cells with phagocytyzed bacilli; D, shadow cells, degenerated cells containing bacilli. Drawn from Leitz oc. 4, obj. 6.

PLATE 45.

FIG. 7. Cells from 9 day culture of splenic tissue from 14 day chick embryo in chicken plasma inoculated with human tubercle bacilli. A represents a loose cluster of bacilli showing marked irregularity in staining; B, a cluster of bacilli and small round cells floating free in serum from culture; C, a shadow cell containing bacilli and a small amount of nuclear substance. B is drawn from Leitz oc. 4, obj. 6, and A and C are drawn from $\frac{1}{12}$ oil immersion.

FIG. 8. Cells from 1 day culture of splenic tissue from 18 day chick embryo in chicken plasma inoculated with human tubercle bacilli. A represents a group of small round cells and parenchyma cells, the latter having phagocytyzed some bacilli; B, a large clump of bacilli with small round cells adherent. Drawn from Leitz oc. 4, obj. 6.

PLATE 46.

FIG. 9. Cells from 7 day culture of splenic tissue from 14 day chick embryo in chicken plasma inoculated with human tubercle bacilli. A represents a parenchyma cell with a single nucleus; B, cells with two nuclei and phagocytyzed bacilli; C, fusion of three mononuclear cells with phagocytyzed bacilli; D, fusion of binuclear and trinuclear cells with bacilli; E, a small fusion multinuclear cell with bacilli. Drawn from $\frac{1}{12}$ oil immersion lens.

FIG. 10. 7 day culture of splenic tissue from 14 day chick embryo in chicken plasma inoculated with human tubercle bacilli, showing the original tissue frag-

ment becoming vacuolated, radiating new growth of connective tissue, and scattered large multinuclear and giant cells in plasma with clumps of bacilli. Small cells and single bacilli are not shown in this low magnification. Drawn from Leitz oc. 3, obj. 1 inch.

PLATE 47.

FIG. 11. Large giant cell from 9 day culture of splenic tissue from 14 day chick embryo in plasma inoculated with human tubercle bacilli, showing phagocytyzed bacilli. Drawn from Leitz oc. 4, obj. 6.

FIG. 12. Large giant cell from 9 day culture of splenic tissue from 14 day chick embryo in chicken plasma inoculated with human tubercle bacilli, showing phagocytyzed bacilli and fragments of small round cells. Drawn from Leitz oc. 4, obj. 6.

FIGS. 5, 6, 7, 9, 10, 11, and 12 are from the same series of cultures. Figs. 6, 9, and 10 are from the same slide; Figs. 7, 11, and 12 are also from one slide.

ABERRANT INTESTINAL PROTOZOAN PARASITES IN THE TURKEY.

By THEOBALD SMITH, M.D.

(From the Department of Comparative Pathology of the Harvard Medical School, Boston, and the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

Plate 48.

(Received for publication, January 11, 1916.)

During a study of tissues from a series of turkeys which had been included in several experiments designed to throw light on the transmission of the protozoan parasite producing the so called blackhead, the writer came upon a case which furnishes the text for this communication. The history of the animal (No. 98) is given in a recent paper (1).

It was raised with eighteen others in an incubator and brooder. No adult turkeys or poultry had been on the grounds for many years. Nine young turkeys from an infected flock, imported July 8, had been kept confined in a distant enclosure. Hatched about May 15, it was well until July 22, when it appeared quiet, sleepy, and refused food. It was chloroformed in the hope of obtaining fresh tissues for histological examination of the intestines. The autopsy showed an absence of blackhead lesions. There was, however, a distinct increase in mucus in the ceca, and several coccidial cysts, measuring about 24 μ by 17 μ, were found in the feces passed during chloroforming. The contents of the duodenum had a markedly yellowish coloration. The muscular tissue of the body was also tinged yellowish. Subsequent examination of tissues fixed in Zenker's fluid showed the presence of peculiar bodies in the subepithelial tissues of the villi of the small intestine, which were tentatively diagnosed as the asexual or schizogonic cycle of some coccidium. The apparently unusual position of the bodies in the adenoid tissue of the mucosa, where they were found in very large numbers, contrasted with the scarcity of the same within the epithelial layer where they, as will be shown

later, were probably not within but between the epithelial cells, induced the writer to give this case a more thorough study.

Coccidia have been generally regarded as exclusively parasites of epithelial cells. The older works of Balbiani (2), von Wasielewski (3), Doflein (4), and others take this for granted. Similarly the investigations by Malassez (5), Labbé (6), Pfeiffer (7), Sjöbring (8), Felsenthal and Stamm (9), Laveran (10), von Wasielewski (11), dealing either with *Eimeria stiedæ* of rabbits or the two known species of avian coccidia (*Eimeria avium* and *Isospora lacazei*) refer only to the epithelial cell parasites.

Metzner (12) was the first to call attention to the presence of coccidia in the subepithelial tissues. He "frequently observed the penetration of *Coccidium cuniculi* into the submucous tissue,[1] regularly into the tunica propria of the cecum, the appendix, and the colon, not infrequently of the small intestine." Metzner promised a discussion of these facts in a subsequent paper which has not, however, appeared.

Fantham (13) mentions the finding of coccidia of grouse in the submucosa, though "much more rarely." He further states that "active schizogony and sporogony go on in the ceca, chiefly in the epithelium, very rarely in the submucosa."

The writer (14), in 1910, described the occasional dislocation of sporonts or gametes of *Eimeria stiedæ* in the rabbit from the epithelial into the subepithelial tissue. This dislocation was ascribed to the transformation of invaded epithelial cells into multinucleated cells followed by disturbance of normal relationship with the adjacent epithelial cells and the subjacent tunica propria. The parasites were not found in the cells of the latter.

Hadley (15) refers to the invasion of the subepithelial tissues of the ceca of turkeys by coccidia. At that time he identified the protozoan parasite of blackhead, which is a purely connective tissue parasite and which does not enter epithelial cells at any time, as a coccidium.

Gérard (16) in describing a coccidiosis of young chicks takes an advanced position on the basis of material studied by him. He states that the schizogonic stage of his parasite goes on in the epithelial and subepithelial tissues and that sporogony goes on only in the epithelium. One of his figures shows many schizonts apparently in the subepithelial cells, but the drawing is evidently not quite true to nature and the large host cells, in certain cases at least, suggest epithelial cells. However, there seems to be enough evidence in his case to indicate extensive

[1] There seems to be much confusion in the use of the terms mucous and submucous tissue. The writer includes in the mucosa the tissue bounded by the muscularis mucosa. Several authors to be quoted have evidently regarded as submucous the subepithelial portion of the mucosa. Metzner in using tunica propria and submucosa as synonymous is using the latter term in this sense.

invasion of the mucous membrane with a slight simultaneous occupation of the epithelium.

It should be stated here that Rivolta (17), as early as 1873, described coccidia-like cysts in the submucosa of fowls which were relatively large and visible to the naked eye as white points, the size of poppy seeds. In 1878 he (18) again referred to similar white points about 0.5 mm. in size, in the same situation in fowls.

In 1893 the writer (19) published a brief account of similar cysts in the villi of the ileum in calves. These were situated near the free end of the villus, 0.3 to 0.4 mm. in diameter, and filled with mobile and immobile merozoites.

Two genera of sporozoa, very different from one another, yet both connecting the coccidia with the gregarinida, should here be mentioned, Léger and Duboscq's (20) *Selenococcidium* and Tyzzer's (21, 22) *Cryptosporidium*. In the former the schizont is a free-living nematode-like organism entering cells only during the periods of asexual and sexual reproduction. The latter passes its entire life cycle attached to, but not in epithelial cells.

Returning to the case of the young turkey, I wish at present to call attention to certain features only, for the sparse material and the absence of a sexual stage make any definite correlation with earlier work and any interpretation premature.

Sections of the intestinal tract were available from the upper, middle, and lower small intestine and the ceca. Conditions as regards the degree of invasion were practically the same throughout the small intestine, but over lymphoid tissue the parasites were scarce.

The epithelium was still present but lifted off from the subjacent core of the villus. The intervening space was filled with a network of circular lines of a precipitate made up of fine granules. The parasites were visible under a low power as vacuoles arranged in an almost continuous band near the margin of the villus core. Occasional bodies were nearer the central axis of the villus (Fig. 1). Under high powers these vacuole-like bodies were found to be but partially empty. A few were well filled. They consisted of some host cell whose cytoplasm had been moulded into a shell (or ring in section) with the much flattened nucleus against this shell. The contents were a very fine lining membrane within which were roundish bodies of various diameters, 2 μ and more, staining feebly reddish and with or without a mass of chromatin. Frequently a body contained two chromatin masses situated at opposite poles, as if division had taken place. Those bodies which were full of the small spheres, contained

about sixteen or more of more or less uniform size (Fig. 2). The vacuolated appearance under low power was due to the disappearance of some or all of the parasitic contents of the host cell. Prolonged search for the characteristic products of asexual multiplication—falciform bodies—brought to light only two or three parasites containing them. It is not to be denied that these may have been moulded into crescent shape by the pressure of the other growing and segmenting members in the same membrane. One of these crescents is shown in Fig. 3.

In addition to the parasites in the core of the villus, a certain number of bodies were found free in the space between villus core (tunica propria of Stoehr) and epithelium. If, as is claimed by most histologists, this space is an artefact, the free parasites must have been embedded in the adenoid tissue at the base of the epithelial cells and set free when the core was pulled away by the shrinking action of the fixing fluid. These bodies differ from those embedded in the core of the villus in having a more condensed cytoplasm, staining more intensely with eosin. They also contain relatively large and more numerous clumps of a substance staining deeply with nuclear dyes and presumably chromatin (Fig. 1 on the left above). These compact clumps are very irregularly grouped and of various shapes, many of them broadly oval or biconvex. No form which may be regarded as normal was detected, no two being precisely alike.

Relatively few parasites were found embedded in the epithelial layer itself. In the one shown in Fig. 2 there is considerable chromatin flattened against the periphery of the body as if it were a host cell nucleus. If so, the body is in a cell lodged between epithelial cells and not in the epithelial cell itself.

The same parasites were also found, but in relatively small numbers, in the mucous membrane of the ceca.

Assuming for the moment that all the parasites were striving toward the formation of merozoites of characteristic form, the great variation in the size of the products of division and the irregularity as regards chromatin, lead the writer to infer that they were largely degenerating forms. This theory is supported by the abortive attempts at repeated multiplication within the primary cysts and the partial disappearance of their contents.

As stated above, it is impossible from this case alone to determine whether the parasite belongs to one or the other of the two well known species of avian coccidia, or whether it is a foreign, aberrant type which fails to survive in the accidental host. The relative smallness of the schizonts, which measure 10 microns, and of the merozoites (the only one that could be found and measured being 5 μ long) led the writer to assume that it is a foreign species. The species described by Gérard has schizonts up to 30 μ by 42 μ, and merozoites 14 μ long (16).

The location of this parasite within cells of the villus indicates that the former may have actively invaded the cells and that phagocytosis did not play a part. The nature of the cells invaded the writer is unable to state.

In general, the invasion of the substance of the villus is of more than ordinary interest to the pathologist. A relatively large amount of foreign proteid is brought within the reach of the absorbents and the disintegration of the parasites may well account for the symptoms produced. Several other young turkeys died within a week after the case under consideration had been chloroformed, and the writer attributed these deaths to arsenic and lead spray. The symptoms in these cases were somnolence, indifference to food, and diarrhea, and the lesions were confined to hyperemia and swelling of the mucous membrane of the small intestines. Coccidia were not found in the intestinal contents or in scrapings of the mucous membrane. Unfortunately tissues of these cases were not saved for microscopic examination. Similarly there was slight temporary indisposition and drooping among other members of the flock, which may have been due to the unknown parasite. The presence of pheasants, sparrows, and song birds in the territory surrounding our experimental grounds may account for the infection, for the young turkeys were allowed to feed over a certain territory outside of the screened shelter each day (1).

Toward the end of the study of this case, the writer came upon two small areas in sections of the ceca of the same case within which nearly every epithelial cell contained minute protozoa which on further study were diagnosed as true coccidia (Fig. 4). The parasites were in an early stage, either as schizonts or sporonts. Putting all the

facts together, the writer is inclined to regard these epithelial cell parasites as belonging to a species distinct from that in the subepithelial tissue, and perhaps the same as the species represented by the oocysts found in the contents of the large intestine at autopsy. To all appearances we have in these parasites true coccidia to deal with.

The invasion of the subepithelial territory of the mucous membrane raises the question concerning the frequency of this phenomenon among coccidia. Is it that this early stage, coming as it does before or with the earliest symptoms, has been overlooked? The negative evidence the writer has cited above from the writings of others should not count too heavily, for much of the work was done by those for whom the life cycle of the parasite was the chief object of pursuit. It may now be desirable to examine the intestines of young healthy animals and of those in which symptoms are just appearing, to determine to what extent early schizogony goes on in the subepithelial tissues, whence direct invasion of epithelial cells for the sporogony may take place from the base of the cells.

There are so many points of difference between the blackhead parasite and the unknown parasite of the mucous membrane as encountered in this single case, that any attempt to present them would require an elaborate restatement of what is now published. Assuming that they are different, we are confronted with the fact that, even after the blackhead parasite shall have been eliminated, the outlook for raising turkeys without some losses due to avian coccidia and perhaps other still unknown protozoan parasites is not very encouraging. Fortunately the mortality due to these aberrant parasites was low. In any case the specific sources of coccidia and other parasites must be found and dealt with.

BIBLIOGRAPHY.

1. Smith, T., Further Investigations into the Etiology of the Protozoan Disease of Turkeys Known as Blackhead, Entero-Hepatitis, Typhlitis, etc., *Jour. Med. Research*, 1915, xxxiii, 243.
2. Balbiani, G., Leçons sur les sporozoaires; recueillies par J. Pelletan, Paris, 1884.
3. von Wasielewski, T., Sporozoenkunde. Ein Leitfaden für Aerzte, Tieraerzte und Zoologen, Jena, 1896.

4. Doflein, F., Die Protozoen als Parasiten und Krankheitserreger, nach biologischen Gesichtspunkten dargestellt, Jena, 1901. Doflein, F., and Koehler, O., Ueberblick über den Stamm der Protozoen, in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, 2nd edition, Jena, 1913, vii, 1.
5. Malassez, L., Notes sur la psorospermose du foie chez le lapin domestique, *Arch. de méd. expér. et d'anat. path.*, 1891, iii, 1.
6. Labbé, A., Sur les coccidies des oiseaux, *Compt. rend. Acad. d. sc.*, 1893, cxvi, 1300.
7. Pfeiffer, R., Beiträge zur Protozoen-Forschung, Berlin, 1892.
8. Sjöbring, N., Beiträge zur Kenntnis einiger Protozoen, *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1897, xxii, 675.
9. Felsenthal, S., and Stamm, C., Die Veränderungen in Leber und Darm bei der Coccidienkrankheit der Kaninchen, *Virchows Arch. f. path. Anat.*, 1893, cxxxii, 36.
10. Laveran, Sur les modes de reproduction d' *Isospora Lacazii*, *Compt. rend. Soc. de biol.*, 1898, l, 1139.
11. von Wasielewski, T., Studien und Mikrophotogramme zur Kenntnis der pathogenen Protozoen, Leipzig, 1904.
12. Metzner, R., Untersuchungen an *Coccidium cuniculi*, *Arch. f. Protistenk.*, 1903, ii, 13.
13. Fantham, H. B., The Morphology and Life History of *Eimeria* (*coccidium*) *avium:* A Sporozoön Causing a Fatal Disease among Young Grouse, *Proc. Zool. Soc., London*, 1910, ii, 672.
14. Smith, T., A Protective Reaction of the Host in Intestinal Coccidiosis of the Rabbit, *Jour. Med. Research*, 1910, xxiii, 407.
15. Hadley, P. B., *Eimeria avium:* A Morphological Study, *Arch. f. Protistenk.*, 1911, xxiii, 7.
16. Gérard, P., Le cycle évolutif d'une nouvelle coccidie aviaire. *Eimeria bracheti* (n. sp.) (*Pfeifferia avium* Labbé (?), *Eimeria avium* Hadley), *Arch. f. Protistenk.*, 1913, xxix, 193.
17. Rivolta, S., and Silverstrini, A., Psorospermose epizootica nei gallinacei, *Gior. d. anat., fisiol. e patol. d. animali*, 1873, v, 42.
18. Rivolta, S., Della gregarinosi dei polli e dell' ordinamento delle gregarine e dei psorospermi degli animali domestici, *Gior. d. anat., fisiol. e patol. d. animali*, 1878, x, 220.
19. Smith, T., Preliminary Notes on a Sporozoön in the Intestinal Villi of Cattle, *U. S. Dept. of Agriculture, Bureau of Animal Industry, Bull. 3*, 1893, 73.
20. Léger, L., and Duboscq, O., *Selenococcidium intermedium* Lég. & Dub., et la systématique des sporozoaires, *Arch. de zool. expér. et gén.*, 1910, series 5, v, 187.
21. Tyzzer, E. E., An Extracellular Coccidium, *Cryptosporidium muris* (Gen. et Sp. Nov.) of the Gastric Glands of the Common Mouse, *Jour. Med. Research*, 1910, xxiii, 487.

22. Tyzzer, E. E., *Cryptosporidium parvum* (Sp. Nov.), a Coccidium Found in the Small Intestine of the Common Mouse, *Arch. f. Protistenk.*, 1912, xxvi, 394.

EXPLANATION OF PLATE 48.

Fig. 1. Section through a villus. The main axis is from above down. Only a few nuclei of the epithelium appear on the left below. The core of the villus is surrounded by a bubbly coagulum under the epithelium. On the right, the marginal zone of the villus contains a row of faintly outlined parasites (from above down). The largest, below, appears as a vacuole containing a few remnants of the schizogonic division. On the left margin of the villus near the bottom of the figure several parasites are faintly outlined.

In the subepithelial (bubbly) space on the left, above, a parasite is seen staining more intensely and with six or more clumps of chromatin.

Fig. 2. A parasite within the epithelial layer. The nucleus of the host cell is shown only in a small part in the photograph. The nucleus of an epithelial cell is seen in the invaded territory crowded towards the free border of the epithelial layer. The progeny of the schizogonic (?) division are faintly outlined. Only one shows a nucleus indistinctly.

Fig. 3. A schizont showing what appears to be a merozoite with a nucleus near one extremity. The schizont is situated in the space between the epithelium and the villus core and is evidently attached to the latter. A row of parasites situated in the marginal zone of the villus is faintly indicated.

Fig. 4. Epithelium of one cecum, showing nuclei near the base of the cells and parasites nearer the free border. The parasite farthest to the right contains four nuclei, but only two are in focus.

A FURTHER STUDY OF THE REGENERATED EPITHELIUM IN CHRONIC URANIUM NEPHRITIS. AN ANATOMICAL INVESTIGATION OF ITS FUNCTION.

By JEAN OLIVER, M.D.

(From the Pathological Laboratory of the Medical School of Leland Stanford Junior University, San Francisco.)

Plates 49 to 52.

(Received for publication, December 20, 1915.)

In a previous report (1) the writer has studied in some detail the changes which follow an acute attack of experimental uranium nephritis and called especial attention to the changes which occur in the epithelial structures of the diseased kidneys. It was pointed out that the regenerative processes differ in a marked degree from those observed following the acute degenerations of other toxic agents, in that the newly formed epithelial cells never assume, or even approximate, the structure of the original renal cells. The cause of this abnormal development was considered to be the deleterious effect of the developing connective tissue, which began at an early period and progressed continuously until the death of the animal from the resulting chronic nephropathy.

That this active proliferation of the connective tissue is lacking with the majority of renal toxic agents is well known, having been confirmed by many authors (Ophüls (2), Dickson (3), Suzuki (4)), and following such degenerations the process of repair follows a different course. Thorel (5) in his studies on regeneration following acute chromium nephritis has shown that there is practically a complete restitution of the degenerated epithelial cells after nine days, though a few tubules with a greater number of nuclei than that seen in the normal tubules may persist for a time in cases of severe damage. This, he states, is the only anatomical lesion persisting for any length of time.

In chronic uranium nephritis, on the other hand, it has been shown that the regenerated cells are easily picked out in the areas of fibrosis as late even as 104 days. The most prominent characteristic of these regenerated cells is the large vesicular nucleus, which is of an oval or irregular shape. A nucleolus is, as a rule, prominent and the chromatin threads are thick and stain heavily with hematoxylin. The "*Kern-Plasma*" relation shows a marked disturbance as compared to the original renal cell. The protoplasm is very scanty, in many cases forming so thin a covering band for the nucleus that it is difficult to see. In other examples, especially in the earlier stages of the process, giant cell-like complexes are seen with a comparatively large amount of protoplasm. This apparent excess of protoplasm is, however, distributed in the later stages over the membrana propria, and the thin covering that it then forms for the tubule is evident.

The question at once presents itself, whether these cells, which anatomically differ so markedly from the original renal cell, are able to functionate in a normal manner. A suggestion that their function is altered is found in the work of Ribbert (6), who has shown that the regenerated cells fail to show the carmine deposits after intravenous administration of the dye, which are seen in the original renal cells. Though these findings were made in the regenerated cells in early stages of repair, the writer has observed that even in the latest stages (54 days) of chronic uranium nephritis, the newly formed cells fail to show the deposits of the dye. Whether this (the presence of vitally stained granules in the cell) may be taken as evidence of secretion, will be considered at a later point of the discussion, though their absence certainly points to an abnormal functional state of some sort in the regenerated cells.

Anatomical changes and their relation to function in the kidney cells have interested observers since the beginning of microscopical investigations. Though no attempt will be made to cover the extensive literature of this subject, for which the reader is referred to monographs on the kidney (Policard (7)), attention is called to certain representative articles.

One group of observers has described vacuolar changes in the renal cells during periods of active secretion. Among these may be noted Gurwitz (8), Lamy, Mayer, and Rathery (9), Enesco (10), and others.

The greater number of investigators, however, have connected changes in the *batonnets* of Heidenhain with variations of function.

The most definite changes in these structures have been observed by Regaud and Policard (11) in the kidneys of the Ophidia. The cells of the kidneys of these animals show a marked variation in the nature of their content, especially in that part of the tubule corresponding with the proximal convoluted tubule of mammals. In some the cells are filled with large granules (*grains de ségrégation*), while in other tubules no granules, but rods are found. Intermediate stages are common. The batonnets in the former type of cell have almost disappeared and exist as short, sinuous filaments localized to the infranuclear zone of the cell, while in the latter forms, *i.e.*, without grains, they are much longer and stretch from the base to the apex of the cell. From the intermediate stages, in which the grains may be seen forming in the batonnets, the authors conclude that there is a direct transformation of the filaments to granules. The filaments, or batonnets, are therefore considered as the agents of elective intussusception for the introduction into the cell of the substances carried in the blood. Analogous findings have been described by Policard (12) in the Batrachians.

In mammals the changes are less definite. Takaki (13), studying the batonnets in the renal cells of the mouse, describes them in resting conditions as homogeneous rod-like formations, which in stages of secretion (feeding of fluids, diuretin administration) change to granules which still preserve the rod-like arrangement of the batonnet. Under pathological conditions somewhat similar changes are described at the outset, which ultimately result in the production of coarse granules of varying size and shape. Kolster (14) and his pupil Hjelt (15) have studied the rabbit kidney with similar result. Enesco finds the same appearances in caffeine and theobromine diuresis.

The greatest objection to the interpretation of all these descriptions of changes in the mitochondrial apparatus as indicative of a normal secretory process, is the fact that similar changes have been described in the study of degenerative processes, especially that known as cloudy swelling. The late stages of this condition, in which large irregular granules are produced, are easily recognized as patho-

logical, but the beginning formation of these granules from the batonnets is apparently identical with the changes described for the process of secretion (Landsteiner (16), Takaki). This fact has led many authors to consider the condition of cloudy swelling as a process of hypersecretion. A further consideration of this phase of the subject will be given in the discussion of the findings of the present investigation.

Though the mitochondrial apparatus of the normal kidney cells, both resting and secreting, and the changes which occur in them in acute degenerations have been carefully studied, so far as we know no descriptions have been given of these structures in the regenerated cells following experimental lesions. As changes in them have been connected with functional variation by so many authors, we have attempted in this investigation to describe their appearance in the regenerated epithelium of chronic uranium nephritis, and have repeated some of the experiments of previous writers on normal kidneys.

We wish to emphasize at this point that there is a marked anatomical difference in the regenerated epithelium in chronic uranium nephritis and those newly formed cells seen after acute experimental nephritides which are not followed by a progressive chronic nephritis (chromium, sublimate). The findings of the present investigation cannot be applied to the latter, as in them the anatomical restitution is complete. From what we know of studies of the acute degenerations in man, there is most likely an equal functional repair. Functional studies of experimental nephritis have been largely confined to the early stages of acute degeneration in which the animal died. Siegel (17) describes a case of uranium nephritis in a dog in which death did not occur for thirty days. In this experiment the secretion of the different urinary constituents decreased until death.

Variations in the Mitochondrial Elements of Normal Kidneys in Different Functional States.

In order to obtain specimens of kidneys in various stages of activity two methods were used. In one set of experiments white rats were given no food or water for three days. In these animals the urinary excretion ceased on the second day as a rule, and little or no urine was found in the bladder when the animal was killed. Another

group of rats was given 2.5 cc. of 5 per cent urea solution intraperitoneally. In some instances this dose was repeated in one hour. The animals as a rule urinated about one hour later, and were killed one hour after the last injection. The fluid in all cases was readily absorbed, and the bladder contained a large amount of urine. The kidneys were removed at once and small pieces fixed in Kolster's fluid which is based on the Regaud technique for showing mitochondrial structures. Thin paraffin sections (3 to 5 μ) were stained with iron-hematoxylin.

Appearance of the Batonnets in Periods of Rest.—In the kidneys of animals which had secreted little or no urine for one or two days, the batonnets presented a strikingly uniform character. In sections of the proximal convoluted tubule which pass at right angles to the lumen of the tubule, they stretch as long, somewhat sinuous rods, from a short distance from the basal membrane to a varying distance towards the lumen of the tubule. As the lumen is very narrow in the resting tubule, the batonnets never reach it, but end at a point about twice the diameter of the nucleus from the membrana propria. Where the section passes obliquely through the tubule, the batonnets are cut into short cylindrical rods, but never appear as isolated round granules.

The ascending limb of the loop of Henle and the distal convoluted tubule are readily distinguished from the above described segment by the relative shortness of their chondrioconts. In these divisions as well, the batonnets exist as homogeneous cylindrical formations, and show much less affinity for the iron-hematoxylin than those of the proximal convoluted tubule.

As it is impossible to place the kidney in a state of absolute rest, one can always find tubules which do not show these characteristic appearances. A few tubules are seen in which the batonnets resemble the descriptions given in the following section.

The Batonnets in Stages of Diuresis.—Though the most apparent variation in the kidneys of rats in stages of diuresis is the dilatation of the lumina of the tubules, certain constant changes are seen in the mitochondrial apparatus of the proximal convoluted tubule. Instead of the long homogeneous structures seen in the resting tubule, the batonnets are now distinctly granular. As well as these small cir-

cumscribed swellings in the rod, isolated granules are also seen, especially in the apical region of the cell. These granules are distinctly round, as compared to the rod-like cut sections of the batonnet seen in the resting tubule, and are of small size, their diameter being slightly greater than that of the original batonnet. The batonnets are, moreover, swollen and much thicker than in the resting kidney. The cell body as well shows this enlargement, the apex of the cell being especially swollen.

The rods of the ascending limb of the loop of Henle and of the second convoluted tubule show no change whatever, but resemble perfectly the appearances seen in the resting kidneys. The less well developed mitochondria of the collecting tubules are also unaffected.

In some instances, in which large amounts of normal salt solution were suddenly injected into the peritoneal cavity, changes were seen which resemble those described in pathological conditions. The droplets were large, even of the diameter of the nucleus, stained irregularly, and were scattered without orderly arrangement. Such appearances were never seen when moderate doses of injection fluid were used.

The Mitochondrial Apparatus in the Regenerated Cells of Chronic Uranium Nephritis.

The kidneys examined in these experiments were those of guinea pigs which had received 3 mg. of uranium and which had been killed at a time when the animal had recovered from the acute effects. Former experiments (1) have shown that the acute lesions are completely replaced by the regenerated cells by the fifteenth day, and as the newly formed connective tissue has not shrunk to any great extent by this time and so obscured the epithelial structures, such a period is most satisfactory for a detailed study of the structure of these cells. As a control other animals were allowed to live until death, one surviving to the eighty-fifth day.

The general structure of the kidney at the fifteenth day of the nephritis has been previously described. The degenerated cells have been entirely replaced by large, irregular epithelial cells, with large oval nuclei, and stand out prominently in the affected areas. The

connective tissue in these regions is still largely cellular, but the beginning collapse of the tubules gives evidence of its shrinkage. In the cortex the effect of this collapse is seen in the dilatation of the proximal convoluted tubule and the glomerular space. A few hyaline casts still persist in the tubules.

In sections stained with iron-hematoxylin after fixation in Kolster's fluid, one is at once struck by the scarcity of the mitochondrial elements as compared to the normal kidney (Fig. 1). With a low magnification the section resembles the appearances seen in vitally stained kidneys. Around each glomerulus is seen a group of tubules which contain the deeply stained batonnets, while the remainder of the cortex is almost entirely free of tubules which possess these heavily stained structures (Fig. 2).

Examination with a higher power shows that the deeply stained groups are Division I of the proximal convoluted tubule.[1] The cells of these divisions are entirely normal in every regard. As the animals had received urea solution intraperitoneally for a purpose to be described later, these tubules show the changes described above as characteristic of a secretory phase. The rodlets are granular and the lumina dilated. More widely scattered through the cortex are seen the distal convoluted tubules and the collecting tubules. These as well are entirely normal, though the latter commonly contain casts, and show no changes in their mitochondrial apparatus.

The most striking appearance, however, is found in the protoplasm of the regenerated cells. Nothing analogous to the batonnet which existed in the original renal cell is seen. In many of the regenerated cells an indefinitely arranged finely granular material is scattered throughout the protoplasm, but never do we see the definite cell organs such as were formed of the mitochondrial substance in the original cells (Fig. 3). In many cases it is difficult to decide if these

[1] The terminology is that of Suzuki, who divides the proximal convoluted tubule into Divisions I, II, and III, according to the amount of vital dye seen in them. Division III is the terminal, and extends into the medulla as far as the boundary of the outer and inner stripe of the outer zone of the medulla. It is this Division III which is affected by uranium and in which most of the reparative processes take place. For further details see Suzuki (4) or the writer's previous article (1).

irregular granules give the reaction of the mitochondria, as they stain so lightly and irregularly. In other cells these granules are more deeply stained, and can then be definitely classed as mitochondria.

Occasionally a tubule is seen which contains batonnets and at the same time evidences of nuclear proliferation. These are intact Divisions I and II of the proximal convoluted tubule, which, as has been described by the writer, often show proliferative tendencies.

The structure of the batonnets and the changes occurring in them in stages of diuresis, differ in no regard from the descriptions of Takaki, and are essentially the same as those figured by Kolster, Hjelt, and Enesco. As compared with the definite changes described in the lower animals, the findings in mammalian kidneys are much less satisfactory. This can perhaps be best explained according to Regaud (18) who says:

> We are therefore led to suppose that in the mammals the phenomena of concentration (in the cells of the tubules) are at a minimum, the morphokinetic variations less marked, and the functioning of the cells continuous, while in the Ophidia the characteristics of function are quite different, phenomena of concentration very important, functioning of the cells discontinuous, alternative and periodic, morphokinetic variations pronounced.

We cannot go into the details of the interesting theory of Regaud and others (Arnold) concerning the relation of the mitochondria (plasmosomes) to cell function, but must refer the reader to the original articles of these authors for the consideration of their deductions. There can be no doubt that the mitochondrial elements of certain divisions of the renal tubule (proximal convoluted tubule) show changes in periods of active diuresis, and that such changes are very limited in periods of comparative rest of the kidney, and are not found in other segments of the tubule.

The relation of these changes to the pathological condition of cloudy swelling is difficult to determine. The two extreme interpretations are, either that the state of cloudy swelling is a manifestation of hypersecretion, or that all changes in the batonnets which result in granule formation are pathological, and both these conceptions have been accepted by eminent investigators. Two facts argue strongly against the latter. First, it is not likely that the injection of a few

cc. of a non-toxic substance (urea or salt) would result in damage to the kidney. Secondly, the formation of granules from the batonnets in the Ophidia and Batrachians occurs without intervention of any sort. It is quite conceivable that the reaction of the cell to a normal functional stimulus and to a weak pathological stimulus may take the same morphological expression, and it is not necessary, in this discussion at least, to draw any wide-reaching conclusions from the scant evidence at hand.

The marked difference which the mitochondria of the regenerated cells in chronic uranium nephritis show in contrast to the original renal cells is a point of special interest. The mitochondria which they possess are similar to the findings in cells of a low degree of differentiation, for in such have been described great variation in amount as well as in the morphological character of these elements. The studies of Lewis and Lewis (19) of the mitochondria in tissue cultures show the wide variations in such proliferating cells. In like manner the mitochondria of the regenerated cell in the kidney vary widely in amount and never show the formation of definite cell organs, the batonnets. While in most epithelial degenerations in the kidney, the regenerated cells, though at first of this embryonic type, later assume a form which differs in no degree from the adult type of the original renal cells (Thorel), in chronic uranium nephritis the early proliferation of the connective tissue or some other factor apparently prevents these epithelial structures from reaching maturity.

We now come to the consideration of the function of these cells in chronic uranium nephritis. As mentioned in the introduction of this article, the absence of dye granules in these cells is suggestive of altered function, though the demonstration of the secretion of some substance native to the normal urine would be much more convincing.

Microchemical tests have been applied to the kidney by many authors, but satisfactory results have been but rarely obtained. Recently Leschke (20) has developed a technique which enables one to demonstrate a large proportion of the urinary constituents in the cells of the renal tubule. Briefly reviewed, he has shown that sodium chloride, urea, phosphates, and uric acid are only present in any practical degree in the cells of the proximal convoluted tubule, including its medullary segment. The glomeruli never show any deposits

of these substances. The finding of the urinary constituents in the cells of the tubules can only be interpreted as evidence of the secretion of these substances into the lumen of the tubule.[2]

This method of anatomically studying the function of the renal cells has been applied to the problem of the function of the regenerated cells in chronic uranium nephritis. As the technique of the various tests is somewhat difficult, urea was selected as representative and demonstrated in the kidneys of the same animals whose kidneys were described in the previous division in which the changes in the batonnets were discussed. We were thus able to observe the morphological changes in secretion by two different methods in the kidneys of a single animal, and so a means of comparison and added control was given.

The Secretion of Urea in the Normal Kidney.

As a confirmation of Leschke's work and as a control on the experiments on nephritic kidneys, several normal animals (guinea pigs) were given different doses of urea solution intraperitoneally. Leschke's technique was followed in detail. It is based on the old quantitative method of Liebig. The kidneys were fixed by injection in a strong solution of mercuric nitrate, washed, and the resulting urea-mercuric nitrate compound reduced by hydrogen sulphide in thin paraffin sections. The method of injection of the fixing solution directly into the artery we found much more satisfactory than fixation in block, as the reagent penetrates very poorly. When counterstained with hematoxylin, the resulting compound, which for convenience we shall refer to simply as urea, shows as a grayish green or brownish black color, depending on its concentration in the cells. As Leschke points out, all protein gives the reaction to a slight degree, but the contrast in the places of deposit of the urea and tissues where no such con-

[2] It is obvious that if one assumed that the substances present in cells of the tubule were absorbed from the lumen, then the concentration of the urine would be lowered by the process. The difficult point to explain by Ludwig's hypothesis has been the large amount of absorption of water necessary to raise the concentration of dilute glomerular urine, so that an assumption of an absorption of the solid constituents would further complicate this theory.

centration can exist (connective tissue) is always sufficient for practical purposes.[3]

The histological appearance of the kidneys of animals which were secreting relatively large amounts of urea, and which were prepared by the method described above, is remarkably constant. An examination of the section with a hand lens shows the divisions of the kidney to be sharply outlined, due to the varying amounts of urea which they contain. The cortex and outer stripe of the outer zone of the medulla are intensely dark, then, considerably lighter, the inner stripe of the outer zone, and finally the comparatively pale inner zone of the medulla.

With a higher magnification it is seen that the darkness of the cortex and the outer stripe of the outer zone of the medulla is due to the loading of the cells of the proximal convoluted tubule with dark granules. The details of the cells are best seen in kidneys where the secretion of urea is at a low concentration, as otherwise the dark granules obscure the finer structures. In such specimens the granules appear as small round bodies which extend from the base of the cell to the apex in definite rows, each granule being connected with its neighbor by a thread-like formation (Fig. 4). When the long axis of the cell is curved, these rows follow the curve of the cell. The apex of the cell shows a definite swelling in this stage, and its protoplasm gives evidence of beginning vacuolization. The cuticular seam is well preserved (Fig. 4).

In kidneys secreting a more concentrated urine the details are less clear. A large number of dark granules fills the entire cell body, obscuring even the nucleus (Fig. 5). The apices of the cells are greatly

[3] *In vitro* a precipitate is formed in the mercuric nitrate solution when sulphates or phosphates are added. These are, however, soluble in acid solution, so that one can prepare a solution of mercuric nitrate, acid with nitric acid, which will throw down the urea alone. As the amount of acid to be added varies with the concentration of mercuric nitrate in the fixative solution, it should be determined by experiment before each injection. Solutions of phosphates and sulphates of a concentration known to be slightly greater than that found in the urine, and a solution of urea roughly approximating that of urine, are prepared. The solution of mercuric nitrate is then acidified with strong nitric acid until the desired point is reached.

swollen and the cuticular seam is no longer evident. The lumen of the tubules contains precipitated masses, and the cut sections of the swollen apices of cells, whose bodies are out of the plane of the section. It is in such stages, in specimens whose fixation is not perfect, that the lumen is transformed into a network of irregular threads from the distortion of these fragile swollen cell apices, and the appearances noted by many authors (Suzuki) after certain fixatives (formalin) are obtained.

Scattered among these heavily stained proximal convoluted tubules are seen the lighter colored distal convoluted and collecting tubules. The latter never show any granules whatever of urea, and though their protoplasm, especially in the large ducts of Bellini, may be rather dark, it is always homogeneous. The distal convoluted tubule and the ascending limb of Henle's loop show a finely granular protoplasm. It was impossible to decide whether these were small granules of urea or not, but they never exist in such amount as to have much practical importance in the secretion of urea. Nor is there the marked variation in these granules depending on the concentration of the urea secretion in the urine, as is seen in the proximal convoluted tubule.

The location of the urea in the normal kidney is essentially that given by Leschke. It has, however, been claimed, in spite of the fairly complete controls which he gave, that the reaction has nothing to do with the urea secretion, but that there is some affinity on the part of the mercuric salt for the proximal convoluted tubule, as all who saw Leschke's preparations granted that the deposits were limited to all practical extent to this segment of the renal tubule. To meet this objection, we have, with the aid of Drs. Addis and Watanabe, combined the study of the urea concentration of the urine with the microchemical demonstration of urea in the renal cells. Guinea pigs were treated as shown in the protocol, the bladder urine was collected at autopsy, and the kidneys were prepared in the manner described above. The urea concentration of the urine was determined by the Marshall urease method by Drs. Addis and Watanabe. Table I gives the details of certain typical examples.

We see that the staining due to the deposit of mercuric salts occurs only in the proximal convoluted tubule and that the amount varies directly and proportionately with the amount of urea secreted in the urine. With these facts determined, we can proceed to the study of the function of the regenerated cells in chronic uranium nephritis.

TABLE I.

Treatment.	Amount of bladder urine.	Amount of urea.	Urea.	Histological appearance of kidney (urea).
	cc.	*gm.*	*per cent*	
Guinea Pig 12. Usual food preceding experiment. 10 cc. 20 per cent urea solution intraperitoneally. 1 hour later killed and bladder urine saved.............	7.7	0.2152	2.79	Very large amounts of urea in proximal convoluted tubule; all details in cells obscured.
Guinea Pig 10. Three days of green food. Water intraperitoneally and killed 1 hour later..	5.0	0.0837	1.674	Moderate amount of urea in proximal convoluted tubule, these plainly darker than ducts of Bellini.
Guinea Pig 11. Same as guinea pig 10....	3.0	0.0179	0.596	Very small amount of urea. Difficult to make out difference in color between proximal convoluted tubule and ducts of Bellini.

The Secretion of Urea by the Regenerated Epithelium in Chronic Uranium Nephritis.

The kidneys examined by Leschke's method were the same as those described above in the study of the changes occurring in the batonnets in chronic uranium nephritis.

With the low power the areas of regenerated cells and fibrosis stand out in the darkly stained cortex, in the medullary rays, and outer stripe of the outer zone of the medulla, as lighter patches (Fig. 6). On more detailed examination the normal Divisions I of the proximal convoluted tubules are found to contain large amounts of urea, and differ in no way from these same segments of the tubule in normal kidneys. The large regenerated cells, however, show a striking difference from the original renal cells in that they show little or no evidence of the urea granules. In some cells, it is true, a few dark isolated granules are seen, but never in a degree even approaching that found in the persisting convoluted tubules. The protoplasm, in the majority of cases, is extremely clear and transparent (Fig. 7). Many of the tubules show collapse, and in these the absence of urea granules is particularly striking. In other tubules the giant cell-like complexes are seen and their protoplasm as well contains no granules. In the medullary rays the persisting segments of the proximal convoluted tubule stand out in marked contrast, as their protoplasm contains the urea in large amount, to the destroyed and regenerated terminal divisions of the proximal convoluted tubule around them.

The confirmation of the findings of other authors of changes in the batonnets of the renal cells in stages of secretion is further supported by the demonstration of a lack of secretion in those regenerated cells which do not contain these cell organs. As we have shown, secretion of urea does not occur in the regenerated cells which do not contain the batonnets, and one might therefore infer that secretion depended on these cell organs.

Another interpretation is, however, possible. It may be that the passage of the urinary constituents occurs by some invisible mechanism, and that the passage of these substances merely disturbs the inactive mitochondria which exist there. This process would be disturbed by some undemonstrable fault in the regenerated cells, which do not contain the mitochondrial elements and which could therefore show no change.

Of these two possibilities the first appears to be a more logical and more direct line of reasoning. As the batonnets are the only cell organs we can possibly connect with such a function, as changes are observed in them in periods of active function, and as function is lacking when they do not exist, it does not seem unreasonable to suppose that function is dependent in large part, if not entirely, upon them.[4]

Another point of interest is the relation of the urea granules and the granules of vital dye to the batonnets. Modern investigators all agree that the dye is secreted at least partially by the proximal convoluted tubule, and that there *Speicherung* of the dye also occurs. Aschoff and his pupils (22) claim that the mitochondria are the seat of this storage, though he denies that there is ever a secretion of the granules themselves. He has further shown that excretion of the dye occurs before the granules are stained, and claims that this is due to the secretion of the dye through the glomerulus in the early periods of elimination. Gross (23), on the other hand, admitting the difference in secretion and *Speicherung* time, claims that this can be explained without recourse to glomerular activity. The granules, during secretion of the dye, take it up from the blood and pass it on to the urine. After considerable time they become overloaded with the dye and then accumulation, or *Speicherung*, begins. As we have never, in a large number (128) of vitally stained animals, seen any deposits of dye (carmine and trypan blue) in the glomerulus, we would accept Gross's explanation as the more likely. Though there are certain differences between *Speicherung* and secretion, the former can only occur when the dye is passing through the cells (secretion),

[4] The observations of Cesa-Bianchi (21) on unfixed renal cells in which he observed changes in the granules with variations in the concentration of the surrounding fluid have been offered as explanatory of the changes in the batonnets in stages of diuresis. The inadequacy of such an explanation is apparent when one considers that if such were the case, the mitochondria throughout the entire length of the tubule would be affected along with the variations in the concentration of the urine, and not a single, comparatively short segment (proximal convoluted tubule) alone.

and can therefore be taken as evidence of a secretory process in the renal cells.[5]

The urea granules also appear in definite rows in suitable specimens, and maintain this arrangement even when the contour of the cell is modified by pressure. To explain this constant appearance we can only imagine the urea to be held by some preexisting structures, and there precipitated by the mercuric salts. If we assume the mitochondria, which apparently play a similar part in the secretion and storage of vital dyes, to be the seat of absorption of the urea from the blood stream, the morphological appearances could be easily explained. The absence of the urea granules from cells which do not contain mitochondrial granules is very suggestive of such an explanation. Such an assumption follows the theory of Regaud who claims for the mitochondria the property of selective absorption of substances destined to be secreted from the blood stream, the condensation of these substances in the granules, and the final elimination of them into the lumen of the gland. It is also essentially the idea of the *Condensatoren* advanced by Gurwitz.

Another point which the present investigation emphasizes is the anatomical and functional differences in the proximal and distal convoluted tubule. The unfortunate use of the common term, convoluted tubule, with the prefix of proximal and distal, has led to a misconception in the relation of these two segments to each other. The current conception, and one seen even in standard texts, seems to be that there is little or no difference, anatomically or functionally, in them. As a matter of fact, the distal convoluted tubule is not a convoluted tubule, but merely the cortical part of the ascending limb of the loop of Henle, which at best has but one or two poorly developed kinks in it. This is best seen in isolated tubules (Peter, Huber, Oliver). Differences in the mitochondria (rods shorter) and absence of a cuticular seam further distinguish it from the proximal convoluted tubule. Functionally the differences are even more marked, for, as we have

[5] We cannot assume the dye to be absorbed from the lumen of the tubules, as did Sobieransky and others, as such an explanation has been rendered impossible by the experiments of Gurwitz.

The reader is referred to Policard's monograph for a detailed discussion of the absorption theory.

shown, dye and urea are not secreted by this segment, nor are changes seen in their batonnets during secretion. The adoption of a more accurate terminology would obviate this confusion.[6]

The final point to which we wish to refer is the relation of failure of secretion of urea on the part of the regenerated cells to the increase of non-protein nitrogen of the blood seen in certain forms of kidney disease. Obviously there can be no direct transference of these conclusions to human nephritis, as the chronic uranium nephritis differs entirely from any known human type of kidney disease. Even in the demonstrated "nitrogen retention" in uranium nephritis many obstacles are met in explaining the faulty elimination by so simple a means. As we have shown, a comparatively large amount of tubule apparently secretes urea normally, so that though the failure of the ability of the regenerated cells to functionate may be one factor, many others, and probably more important ones, must be considered, as Mosenthal (24) has shown. It is hoped, however, that these newer methods of morphological investigation may lend them-

[6] The French and German terms, in comparison with the English, are as follows:

French (Policard).	English.	German (Peter).
I. Segment à striation et à cuticle (tube contourné).	Proximal convoluted tubule.	Hauptstück (Konvolut).
Partie corticale.		Pars convoluta.
Partie medullaire.		Pars recta.
II. Segment grêle.	Descending limb of Henle's loop.	Absteigender, dünner Schenkel der H. Schleife.
III. Segment intermédiaire (à striation sans cuticle).		Aufsteigender, dicker Schenkel der H. Schleife.
Partie medullaire.	Ascending limb of Henle's loop.	
		Zwischenstück.
Partie corticale.	Distal convoluted tubule.	Eigentliches Schaltstück.
IV. Segment dit excréteur (sans striation ni cuticle).	Collecting tubule.	Sammelrohr.

selves to combination with functional studies of the kidney, and that by such a combination some light may be cast on the more obscure side of the problems of kidney function, both normal and in disease.

SUMMARY.

1. Definite morphological changes occur in the batonnets of the proximal convoluted tubule in stages of activity.
2. Atypical mitochondria but no batonnets exist in the regenerated cells found in chronic uranium nephritis.
3. No secretion of urea occurs in these cells which do not contain the cell organs.
4. The urea is secreted normally in the proximal convoluted tubule only (Leschke).
5. The urea appears in the form of granules, which are arranged in definite rows.
6. It is suggested that the secretion of the urea is by means of the mitochondrial batonnets which act as condensors.

I take this opportunity to thank Dr. Ophüls for his interest and frequent aid in these investigations, and Drs. Addis and Watanabe for their determinations of the urea content of the urine in several experiments.

BIBLIOGRAPHY.

1. Oliver, J., The Histogenesis of Chronic Uranium Nephritis with Especial Reference to Epithelial Regeneration, *Jour. Exper. Med.*, 1915, xxi, 425.
2. Ophüls, W., Experimental Chronic Nephritis, *Jour. Am. Med. Assn.*, 1907, xlviii, 483.
3. Dickson, E. C., A Report on the Experimental Production of Chronic Nephritis in Animals by the Use of Uranium Nitrate, *Arch. Int. Med.*, 1909, iii, 375.
4. Suzuki, T., Zur Morphologie der Nierensekretion unter physiologischen und pathologischen Bedingungen, Jena, 1912.
5. Thorel, Ch., Pathologisch-anatomische Beobachtungen über Heilungsvorgänge bei Nephritis. Eine experimentelle und kritische Studie, *Deutsch. Arch. f. klin. Med.*, 1903, lxxvii, 29, 395, 470.
6. Ribbert, H., Die Abscheidung intravenös injizierten gelösten Karmins in den Geweben, *Ztschr. f. allg. Physiol.*, 1904, iv, 201.
7. Policard, A., Le tube urinaire des mammifères, *Rev. gén. d'histol.*, 1908, iii, 307.

8. Gurwitz, A., Zur Physiologie und Morphologie der Nierenthätigkeit, *Arch. f. d. ges. Physiol.*, 1902, xci, 71.
9. Lamy, H., Mayer, A., and Rathery, Fr., Études sur la diurèse. IV. Modifications histologiques du rein au cours de l'élimination de l'eau et des cristalloïdes, *Jour. de physiol. et de path. gén.*, 1906, viii, 624.
10. Enesco, I., Contribution à l'étude histo-physiologique de la cellule du tube contourné et de l'anse ascendante de Henle chez quelques mammifères, sous l'influence des substances diurétiques. Première note, *Compt. rend. Soc. de biol.*, 1913, lxxiv, 914; Contribution à l'étude histo-physiologique de la cellule rénale chez quelques mammifères sous l'influence des substances diurétiques. Deuxième note, 973.
11. Regaud, C., and Policard, A., Recherches sur la structure du rein de quelques ophidiens, *Arch. d'anat micr.*, 1903, vi, 191.
12. Policard, A., Le tube urinaire des mammifères, *Rev. gén. d'histol.*, 1908, iii, 368.
13. Takaki, K., Ueber die Stäbchenstrukturen der Niere, *Arch. f. mikr. Anat.*, 1907, lxx, 245.
14. Kolster, R., Mitochondria und Sekretion in den Tubuli contorti der Niere. Eine experimentelle Studie, *Beitr. z. path. Anat. u. z. allg. Path.*, 1911, li, 209.
15. Hjelt, K. J., Über die Mitochondria in den Epithelzellen der gewundenen Nierenkanälchen bei der Einwirkung einiger Diuretika (Koffein und Theocin), *Virchows Arch. f. path. Anat.*, 1912, ccvii, 207.
16. Landsteiner, K., Ueber trübe Schwellung, *Beitr. z. path. Anat. u. z. allg. Path.*, 1903, xxxiii, 237.
17. Siegel, W., Ein Stoffwechselversuch bei Urannephritis am Hunde, *Ztschr. f. exper. Path. u. Therap.*, 1907, iv, 561.
18. Regaud, C., Les mitochondries organites du protoplasma considérés comme les agents de la fonction eclectique et pharmacopexique des cellules, *Rev. de méd.*, 1911, xxxi, Special No., 681.
19. Lewis, M. R., and Lewis, W. H., Mitochondria (and Other Cytoplasmic Structures) in Tissue Cultures, *Am. Jour. Anat.*, 1915, xvii, 339.
20. Leschke, E., Untersuchungen über den Mechanismus der Harnabsonderung in der Niere, *Ztschr. f. klin. Med.*, 1914, lxxxi, 14.
21. Cesa-Bianchi, D., Experimentelle Untersuchungen über die Nierenzelle, *Frankfurt. Ztschr. f. Path.*, 1909, iii, 461.
22. Aschoff, L., Zur Frage der kadaverösen Autolyse, *Verhandl. d. deutsch. path. Gesellsch.*, 1914, xvii, 113.
23. Gross, W., Über den Zusammenhang zwischen Farbstoffausscheidung und vitaler Färbung der Nieren, *Verhandl. d. deutsch. path. Gesellsch.*, 1914, xvii, 123.
24. Mosenthal, H. O., Nitrogen Metabolism and the Significance of the Non-Protein Nitrogen of the Blood in Experimental Uranium Nephritis, *Arch. Int. Med.*, 1914, xiv, 844.

EXPLANATION OF PLATES.

Plate 49.

Fig. 1. Normal kidney of a guinea pig fixed in Kolster's fluid and stained with iron-hematoxylin to show the batonnets. At the left is seen a glomerulus and around it are the sections of the proximal convoluted tubule which are filled with the long batonnets. Bausch and Lomb obj. 1/6, oc. 1.

Fig. 2. Kidney of a guinea pig on the 15th day of uranium nephritis. Fixation and staining as above. At the upper edge of the figure is seen a glomerulus, and below, six sections of the normal Division I of the proximal convoluted tubule, which contain the batonnets. A normal collecting tubule at A does not contain these structures. The remainder of the tubules are lined with regenerated epithelium, which does not possess any of the batonnets. The large irregular nuclei and indefinite lumen of the regenerated tubule are shown at B. Bausch and Lomb obj. 1/6, oc. 1.

Plate 50.

Fig. 3. A tubule with regenerated epithelium from Fig. 2. The large irregular nuclei and indefinite lumen are well seen. In the protoplasm of the cells there are many poorly staining granules but no definite batonnets. In the lower left corner is a collecting tubule which normally does not possess the rodlets. Note the difference in size and shape of the normal nuclei in this tubule and those of the regenerated tubule. Bausch and Lomb obj. 1/12, oil immersion, oc. 1.

Fig. 4. Kidney of a guinea pig in the stage of urea secretion at low concentration. Leschke preparation. Two terminal divisions of the proximal convoluted tubule. The cells are covered with a definite cuticular seam. The protoplasm is filled with fine granules arranged in rows and connected by fine threads. The apices of the cells are swollen and beginning vacuolization is seen in them. At the left the cut sections of such cell apices fill the lumen. Bausch and Lomb obj. 1/12, oil immersion, oc. 1.

Plate 51.

Fig. 5. Low power of kidney of a guinea pig in the stage of secretion of urea at high concentration. The beginning of Division I of the proximal convoluted tubule and the lower sections which lie around the glomerulus are seen. All these tubules are so filled with the urea granule as to obscure all detail, even the nuclei being hidden. The lumina of the tubules contain the poorly fixed cell apices and granular material. Bausch and Lomb obj. 1/6, oc. 1.

Fig. 6. Kidney of a guinea pig on the 15th day of uranium nephritis. The animal was given urea solution and kidney prepared by Leschke's technique. On either side of the section are seen glomeruli and surrounding them the normal Divisions I of the proximal convoluted tubule, which are filled with the dark staining urea. The central part of the figure is filled with the regenerated tubules, which contain practically no urea granules. On close examination the irregular nuclei of the regenerated tubules is evident. Bausch and Lomb obj. 2/3, oc. 1.

Plate 52.

Fig. 7. High power of a group of tubules of Fig. 6. Three cross-sections and one longitudinal section of the normal Divisions I of the proximal convoluted tubule are seen filled with varying numbers of urea granules. The connective tissue proliferation around these tubules is evident. The regenerated tubules A are filled with irregularly shaped cells, which are in excess of the normal number, and contain practically none of the urea granules. Bausch and Lomb obj. 1/6, oc. 1.

STUDIES ON TREPONEMA PALLIDUM AND SYPHILIS.

II. Spirochæticidal Antibodies Against Treponema Pallidum.

By HANS ZINSSER, M.D., and J. G. HOPKINS, M.D.

(*From the Department of Bacteriology of the College of Physicians and Surgeons, Columbia University, New York.*)

(Received for publication, December 30, 1915.)

In a previous communication we have reported on the agglutinins which are formed in rabbits following systematic inoculation with concentrated culture material of *Treponema pallidum*. Incidentally it may be stated that a sheep has also been successfully used. The antigen used for the immunization of these animals was similar to that employed in the first agglutination experiments, except that we have lately used cultures grown on a medium devised by Miss Gilbert in this laboratory containing egg and animal sera instead of tissue and animal sera. The egg cultures which have been used have been made up in a manner similar to that employed in the preparation of Dorset's egg, the egg broth mixture being coagulated in the inspissator, with or without glycerine, in high tubes, the broth serum mixtures poured into the tubes, and these sealed with sterile oil after inoculation. We mention this medium in passing since it promises to furnish an excellent method for obtaining clean antigen, unmixed with tissue detritus, a disadvantage incident to tissue cultures. A subsequent report will be made on this medium. The inoculations in animals have always been made in such a way that cultures containing sheep serum were used for immunization, and the cultures finally used in reaction with the immune serum were grown on ascitic fluid. When tissue cultures were employed, rabbit kidney was used in both cases. The reason for this was that we wished to avoid possible protein-antiprotein reactions which by fixing complement would render our experiments negative.

The spirochæticidal experiments were carried out according to the following general scheme. A series of high tubes, such as are used for treponema cultivation, were prepared, and into them equal quantities of salt solution emulsions or culture fluid emulsions of the treponemata were placed. At first experiments were duplicated with various dilutions of the spirochæte emulsions in order to be sure that we did not use too large a quantity of the microorganisms, an accident which might entirely defeat the possibility of a positive result, in that incomplete killing could not be differentiated from absolute failure to kill. Later we found that emulsions so made up that 10 to 15 spirochætes per dark-field were visible were suitable for the work, and after this, such emulsions in 0.1 cc. quantities, or weaker emulsions in twice this amount, were used throughout.

To the treponemata in these tubes normal serum and immune serum in various dilutions, heated and unheated, and immune serum, inactivated and reactivated with normal serum, were added. Controls with salt solution were always done, and in all cases the experiments were set up in duplicate, sometimes in triplicate sets, in order to obviate the possibility of an occasional negative plant. The total volumes were brought up to 1 cc. in every case, and these tubes were set away in the thermostat at 37.5°C. for 2 to 3 hours. At the end of this time a bit of sterile kidney tissue and ascitic agar were added to each of the tubes, the medium was covered with liquid petrolatum, and the tubes were incubated. Readings were made at the end of from 2 to 3 weeks when the controls had shown active growth.

No experiment was accepted in which the controls were not well developed and in which the duplicates did not check up. Both immune rabbit serum and sheep serum were used in individual experiments.

In Experiment I we submit simply the fact that normal rabbit serum in quantities of 0.1 cc. does not kill the spirochætes, whereas a relatively weak immune serum we had at that time did so, and that spirochæticidal action was also evident where the inactivated immune serum was reactivated with an ineffective dose of normal active serum.

EXPERIMENT I.

Feb. 12, 1915.

Tube No.	Serum.	Amount.	Salt solution.	Spirochæte suspension.	Result.
		cc.	*cc.*	*cc.*	
1 (CIa).	Normal rabbit serum, unheated.	0.1	0.9	0.1	Excellent growth.
2 (CIIa).	Immune rabbit serum, inactive.	0.1			
	Normal rabbit serum, active.	0.1	1.8	0.1	No growth.
3 (CIIIa).	Immune rabbit serum, active.	0.1	0.9	0.1	" "
4 (D).	Salt solution.		1.0	0.1	Growth.

In Experiment II it is apparent that normal serum in quantities of 0.04 cc. no longer exerted spirochæticidal action. Immune serum exerted some spirochæticidal action in this quantity, in that no growth whatever was found in one of the series, and in the other after considerable search a few were found which we judged to be probably remains of those planted, since we know from other experience that spirochætes may remain unchanged for a long time without growing in tubes so prepared, but for the sake of not falling into possible error we listed this as doubtful growth. It is apparent, however, from Tubes DDII that immune serum in quantities of 0.01 cc., reactivated with an amount of normal serum which by itself had no inhibitory action, gave a sterile tube, which therefore indicates the spirochæticidal action in the reactivated serum. The same thing, less strikingly (since there was growth in one of the tubes of the set), is true of Tubes EEII, in which 0.005 cc. of immune inactivated serum was used.

In the preceding experiment sheep serum, normal and immune, was used, and, as agglutination has shown, the sheep had not responded as actively as did rabbits in antibody formation, probably since it had not been easy to get relatively as large amounts into the sheep up to that time as was possible in the case of a rabbit.

In the following experiments normal rabbit and immune rabbit sera were used.

EXPERIMENT II.

Serum from a normal sheep and from a sheep immunized with Culture A were used, according to the following protocol, dilutions being made in salt solution so that the total volume in each tube was 1.0 cc. The inactivated sera were heated at 56°C. for a half hour before dilution. 0.2 cc. of a suspension of spirochætes of Culture A, containing 4 to 6 organisms to the field, was added to each tube and the tubes were incubated for 2 hours at 37.5°C. A piece of sterile kidney tissue and ascitic agar were then added to each tube, the medium was covered with liquid petrolatum, and the tubes were incubated. The experiment was set up in duplicate, and the results on the duplicate tubes are recorded separately.

Oct. 20, 1915.

Tube No.	Serum.	Amount.	Result.	
			Series A.	Series B.
		cc.		
1 (AI).	Normal sheep serum, active.	0.1	Growth.	Growth.
2 (AII).	Immune serum, active.	0.1	No growth.	No growth.
3 (BI).	Normal " "	0.04	Growth.	Growth.
4 (BII).	Immune " "	0.04	" (?)	No growth.
5 (CI).	Normal " "	0.02	"	Growth.
6 (CII).	Immune " "	0.02	"	"
7 (CIII).	Normal " "	0.01	"	"
8 (DDI).	" " inactive. " " active.	0.01 0.02	"	"
9 (DDII).	Immune " inactive. Normal " active.	0.01 0.02	No growth.	No growth.
10 (DDx).	Immune " inactive. Normal " active.	0.01 0.01	Growth.	Growth.
11 (EEI).	" " inactive. " " active.	0.005 0.02	"	"
12 (EEII).	Immune " inactive. Normal " active.	0.005 0.02	"	No growth.
13 (EEx).	Immune " inactive. Normal " active.	0.005 0.01	"	Growth.
14 (F).	Salt solution control.	1.0	"	"

In Experiment III it will be seen that while the spirochæticidal action of the normal serum was eliminated below 0.1 cc., no growth occurred in the tubes containing as little as 0.02 cc. of the immune serum.

EXPERIMENT III.

Immune rabbit serum, No. 1 (Culture A used for immunization), and normal rabbit serum were employed. The culture used was one of the homologous Strain A.

Oct. 25, 1915.

Tube No.	Serum.	Amount.	Result.	
			Series A.	Series B.
		cc.		
1 (AI).	Normal serum, active.	0.1	No growth.	No growth.
2 (AII).	Immune " "	0.1	" "	" "
3 (BI).	Normal " "	0.04	Growth.	Growth.
4 (BII).	Immune " "	0.04	No growth.	No growth.
5 (CI).	Normal " "	0.02	Growth.	....
6 (CII).	Immune " "	0.02	No growth.	No growth.
7 (F).	Salt solution.		Growth.	Growth.

Experiment IV illustrates the fact that the spirochæticidal action of a rabbit immunized with our Strain A was equally active against one of the Noguchi cultures, known as N_3 in our laboratory. In this experiment again immune serum, active, inhibited in quantities as small as 0.04 cc., whereas normal serum did not prevent growth

EXPERIMENT IV.

Immune Rabbit, No. 1; normal rabbit serum; Culture N_3.

Nov. 17, 1915.

Tube No.	Serum.	Amount.	Result.	
			Series A.	Series B.
		cc.		
1 (AI).	Normal serum, active.	0.1	Growth.	Growth.
2 (AII).	" " inactive.	0.1	"	"
3 (AIII).	Immune " active.	0.1	No growth.	No growth.
4 (AIV).	" " inactive.	0.1	Growth.	Growth.
5 (BI).	Normal " active.	0.04	"	"
6 (BII).	Immune " "	0.04	No growth.	No growth.
7 (BIII).	" " inactive.	0.04	Growth.	Growth.
8 (C).	" " "	0.1		
	Normal " active.	0.02	No growth.	No growth.
9 (E).	" " "	0.02	Growth.	Growth.
10 (F).	Salt solution.		"	"

in quantities of 0.1 cc. Moreover, immune serum, inactivated, in quantities of 0.1 cc. reactivated with 0.02 cc. of normal active serum, resulted in complete absence of growth. The additional Tube 7 (BIII) showed that inactivation of the immune serum prevented its spirochæticidal action, showing that the structure of the spirochæticidal bodies is similar to that of the bactericidal bodies known in the case of other microorganisms.

SUMMARY AND CONCLUSIONS.

We believe that our experiments have shown that the serum of rabbits and sheep immunized with cultures of *Treponema pallidum* acquires spirochæticidal properties for these culture spirochætes.

The normal serum of these animals also possesses spirochæticidal action if used in sufficient quantities, and the action of the immune serum represents probably an increase of the antibodies normally present.

Both normal and immune spirochæticidal properties are destroyed by heating to 56°C.

The spirochæticidal action of the immune serum can be reactivated by the addition of fresh normal serum of the same species, insufficient in amount to exert a spirochæticidal effect by itself.

The structure of these spirochæticidal bodies, therefore, is entirely analogous to that of the well known bactericidal antibodies known to exist in antibacterial sera.

We do not wish to have these results interpreted as applying to virulent spirochætes as well as to culture spirochætes. A subsequent publication will demonstrate why we specify this at present.

STUDIES ON TREPONEMA PALLIDUM AND SYPHILIS.

III. The Individual Fluctuations in Virulence and Comparative Virulence of Treponema Pallidum Strains Passed through Rabbits.

By HANS ZINSSER, M.D., J. G. HOPKINS, M.D., and MALCOLM McBURNEY, M.D.

(*From the Department of Bacteriology of the College of Physicians and Surgeons, Columbia University, New York.*)

(Received for publication, December 30, 1915.)

In the course of the experimental work carried out at this laboratory in connection with antibody formation against *Treponema pallidum*, it has been necessary to keep a number of strains of this microorganism alive, in a virulent condition, by passage through rabbits. Incidental to this work we have felt it desirable to study fluctuations in virulence in individual strains and to determine, if possible, whether there is a variation in virulence between races of treponema obtained from different human sources.

A great deal of work has been done on rabbit syphilis, the most extensive study probably being that of Uhlenhuth and Mulzer.[1] These writers observed a distinct increase of virulence in the course of rabbit passage. They found that after the fourth passage they often had 69 per cent to 70 per cent of takes, which rose to a frequent 100 per cent after the 11th or 12th generation, with a coincident progressive tendency for generalization.

Noguchi,[2] in doing his earlier work on rabbit syphilis, noticed a distinct difference in the morphology of various strains of treponemata in that some strains were conspicuously thinner than others, and that this morphological difference has some direct correlation to ease of cultivation, agility of motion, and infectiousness. He describes three forms: a thin one with an average width of 0.2 of a micromillimeter, a thick form with an average thickness of 0.3 of a micromillimeter, and an intermediate one measuring 0.25 of a micromillimeter. He states that the thicker types do not produce any changes for 5 to 6 weeks and then

---

[1] Uhlenhuth, P., and Mulzer, P., *Arb. a. d. k. Gsndhtsamte.*, 1913, xliv, 307.

[2] Noguchi, H., *Jour. Exper. Med.*, 1912, xv, 201.

result in hard, indurated nodules which are sharply demarcated, while the thinner ones usually have a shorter incubation time, producing a swelling of the testicle in 10 to 14 days, which by gradual swelling is transformed into a large diffuse lesion. Nichols[3] has studied a strain derived from the central nervous system; that is, from spinal fluid in a case of nervous relapse following salvarsan. It corresponded to the thick form of Noguchi, and Nichols confirms the observation that this type produces hard cartilaginous nodules. He states that the lesions developed by his strain were easily differentiated from the soft, edematous processes produced by the strains obtained from chancres and mucous patches, and calls attention to the fact that patients with nervous relapse often show papular rather than macular secondary eruptions. He states that his strain had a characteristic localization on the scrotal side of the tunica vaginalis and was characterized by a great tendency to generalization, in that it produced chancres on the opposite scrotum, nodules in the opposite testis, keratitis, and nodules on the eyelids, which developed after an unusually short incubation time. It was Nichols' impression that *Treponema pallidum* strains may vary extensively in their invasive powers, and that a certain amount of prognostic deduction can be drawn from a study of the rabbit pathogenicity of a strain isolated from any given case.

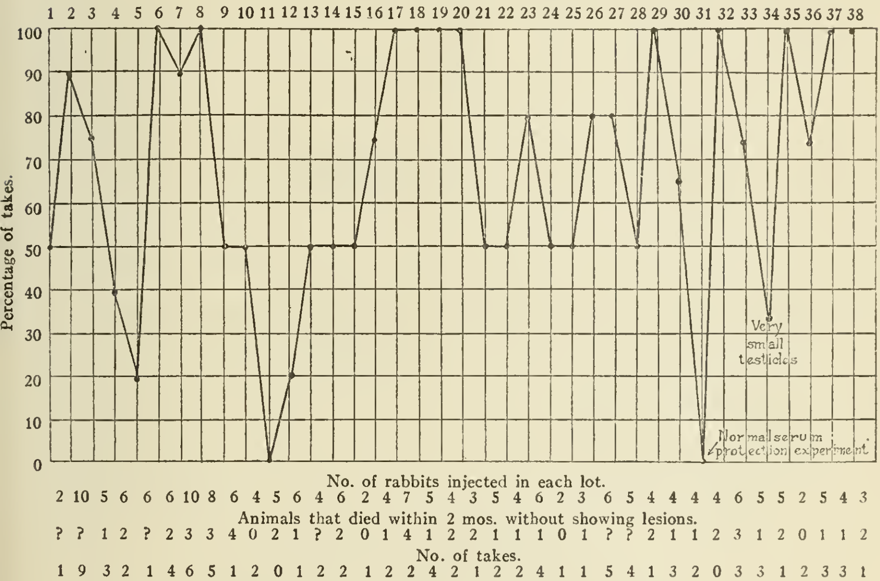
These observations are, of course, of great importance for the knowledge of the biology of these microorganisms and might have serious bearing upon the clinical and immunological comprehension of syphilis. We have therefore included a comparative study of the strains that we have used incidental to our work in other fields of observation in the hope of throwing further light upon these relations.

We have inoculated rabbits altogether from fourteen human cases, none of them nervous system cases, about half of them from chancres, and the rest from mucous patches and condylomata. We were successful in starting the strains and carrying them to the second generation in six of these cases. One of these, Strain O, was lost after the third generation; another, Strain K, obtained from the New York Department of Health, was lost after ten generations. The other four have been carried along up to the present day and are now in generations varying from the twelfth to the twenty-first. Strain T, which we have run in addition to these, is Dr. Nichols' strain, which he kindly sent to us in his own seventeenth generation, and which has now gone through an additional nine generations in our hands.

[3] Nichols, H. J., *Jour. Exper. Med.*, 1914, xix, 362.

Text-figs. 1 to 5 represent fluctuations in percentage takes of Strains A, T, F, L, and S. The dots on the lines, marking angles, represent not generations, but groups of rabbits inoculated at one time, since often two or three lots of animals were inoculated from various lesions in a single generation. The total number of generations of each strain is given in Arabic numerals in the heading for each chart. The figures at the bottom of the charts represent the number of

Strain A, 21 Generations.

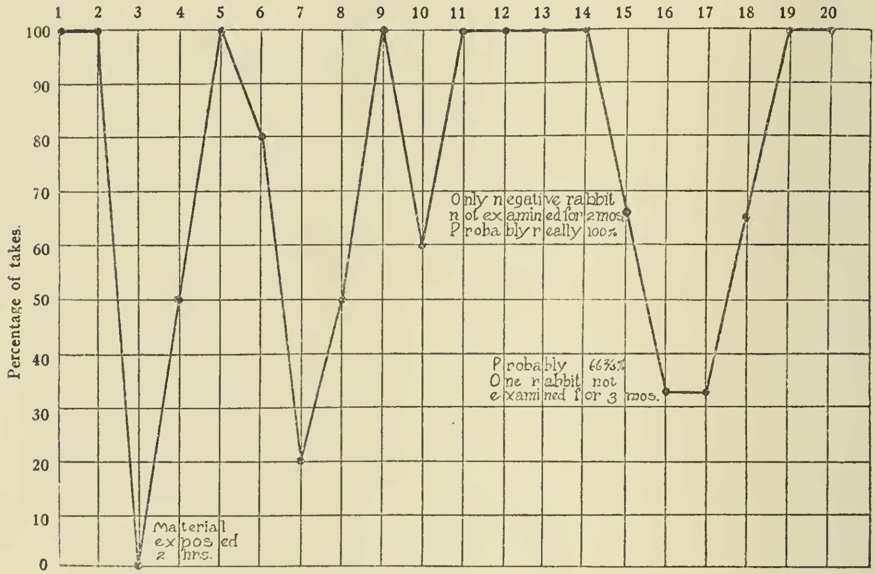


Text-Fig. 1. Chart showing positive reaction in successive generations of Strain A.

rabbits injected in each lot in the uppermost row, and the number of takes in the lowest row. The middle row represents animals which died within 2 months without showing lesions. Since in the earlier part of our work we were unable to prove out by puncture every lesion, we have omitted from our statistical percentages all doubtful lesions which might have been abscesses and were not proved microscopically, and we have also disregarded in the statistics all rabbits that died within 2 months without showing lesions. It is not improb-

able that *Treponema pallidum* can remain latent for long periods in the testicle without either growing or dying out, and for some reasons, not entirely clear, can later enter into a sudden phase of growth by which a lesion is produced. The work was rendered difficult also (and by this we account for the great irregularities in the charts in certain places) by the fact that in the course of these experiments,

Strain F, 15 Generations.



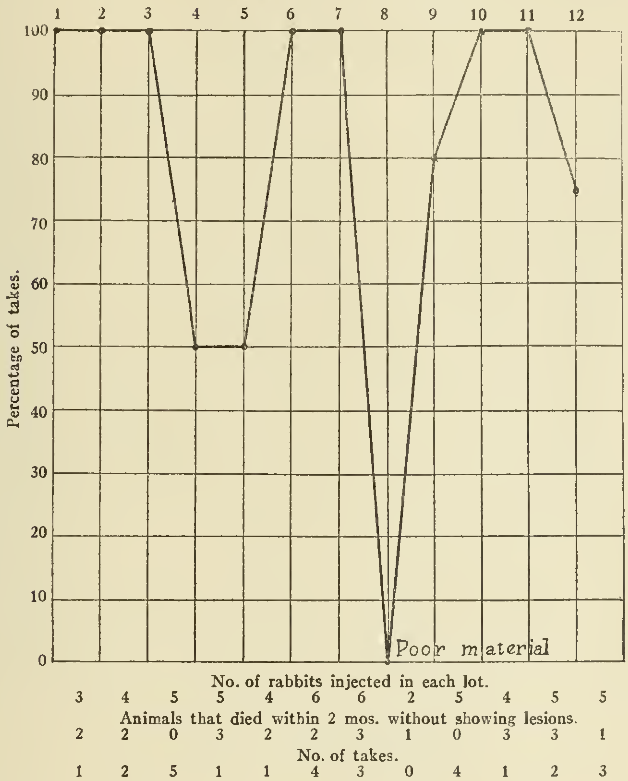
No. of rabbits injected in each lot.	4	4	10	6	3	5	5	4	4	6	4	4	4	4	5	5	5	5	3
Animals that died within 2 mos. without showing lesions.	0	0	?	2	1	0	1	2	1	1	2	2	2	1	2	2	2	2	0
No. of takes.	4	4	0	2	2	4	1	1	3	3	2	2	2	3	2	1	1	2	3

TEXT-FIG. 2. Chart showing positive reaction in successive generations of Strain F.

which have covered a period ranging from October, 1913, up to the present time, there occurred occasional epidemics of bacterial infection which killed a large number of rabbits. Often also rabbits of unsuitable size with very small, undeveloped testicles were the only ones that could be procured. Furthermore, on a number of occasions, the material for injection was not the best, and this too tended to make great differences in the number of takes obtained.

When one studies these charts first, without qualifying considerations, in regard to the percentages of takes, the following facts become apparent.

Strain L, 12 Generations.



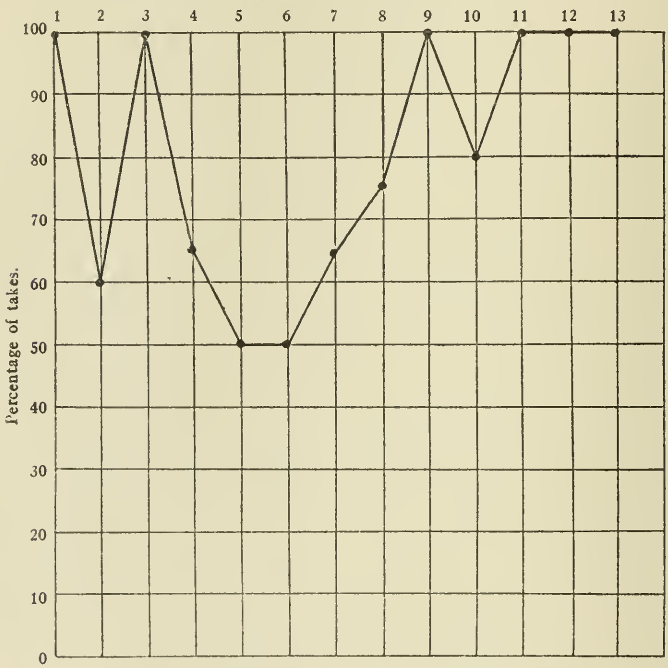
TEXT-FIG. 3. Chart showing positive reaction in successive generations of Strain L.

Strain A.—One of the rabbits inoculated developed a lesion in the first generation, which, of course, though technically 50 per cent, means nothing considering the small number of rabbits, but in the second generation 90 per cent of the rabbits became syphilitic. In the next generation only 20 per cent, then in quick succession two lots with 100 per cent lesions were found, and after a number of gener-

ations in which in one lot we obtained no takes whatever, again 100 per cent of the rabbits in four successive inoculations were positive.

It is not necessary to specify individually the other charts since throughout all of them it is apparent that 100 per cent of takes often occur early in the passage of the strain, which returns again later

Strain S, 12 Generations.



No. of rabbits injected in each lot.

2 6 6 5 2 4 4 4 4 5 5 4 3

Animals that died within 2 mos. without showing lesions.

1 1 2 2 0 2 1 0 2 0 2 1 2

No. of takes.

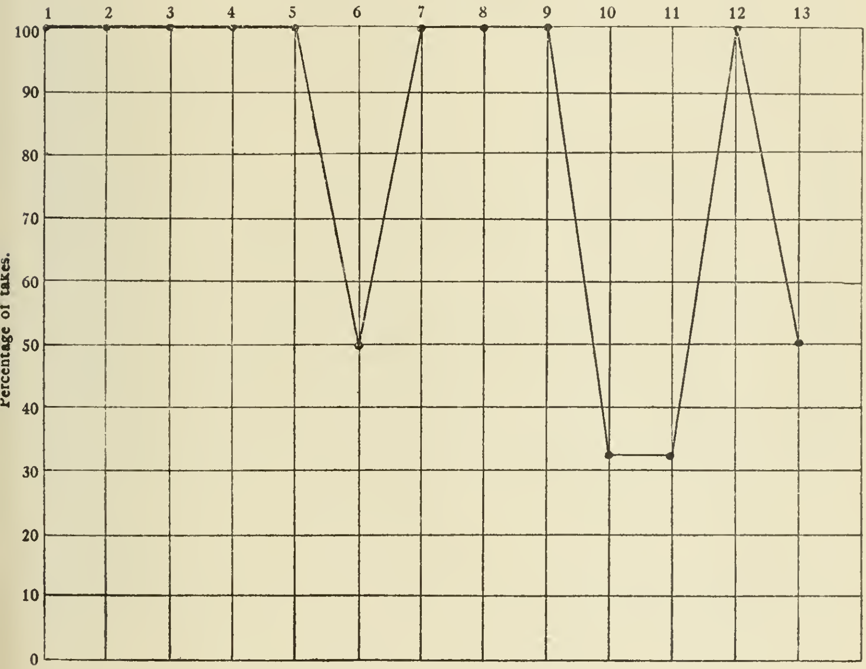
1 3 4 2 2 1 2 3 2 4 3 3 1

TEXT-FIG. 4. Chart showing positive reaction in successive generations of Strain S.

with fluctuations between, and it is our impression in studying in detail the long series of rabbit inoculations that we have observed, that there is neither a rise nor fall in the virulence of the strains studied by us, but that an originally high virulence possessed by treponema derived from the human body for rabbits, remains high and fluctuates

only according to other factors which we shall discuss later. We do not believe that there is a gradual increase of virulence, as stated by Uhlenhuth and Mulzer, but that these strains remain about the same throughout succeeding generations. Indeed, we believe that with

Strain T, 9 Generations.



No. of rabbits injected in each lot.												
7	3	5	2	4	4	4	5	5	4	4	4	3
Animals that died within 2 mos. without showing lesions.												
5	0	2	0	2	2	3	3	4	1	1	0	1
No. of takes.												
2	3	3	2	2	1	1	2	1	1	1	4	1

TEXT-FIG. 5. Chart showing positive reaction in successive generations of Strain T.

the most favorable conditions for inoculation after the first inoculation from man, when it can be obtained pure and can be injected into suitable rabbits, with favorable technique and in sufficient quantity, 100 per cent of takes, or very nearly that, would be the rule, at least in all the strains studied by us.

The factors upon which we believe that the percentage of takes depends are: (1) The quality of the material. When we have had large diffuse lesions (which are spoken of by most writers as edematous, but in which the soggy, soft mass is filled with a gelatinous, sticky exudate), in which large numbers of treponemata can be obtained in the press juice, which can be injected without much tissue detritus, the chances of obtaining a high percentage of takes are excellent. (2) The type of rabbit injected. We do not believe that the race or color of the rabbit has much to do with its susceptibility to syphilis, since we have seen no difference in this regard. We do believe that intratesticular inoculation is more successful when we have been able to get well grown rabbits with relatively large testicles in which it has been easy to inject directly into the testicular substance and diffuse the material throughout the testicle while slowly pulling out the needle. When small rabbits with undeveloped testicles were used and a good deal of the material has gone into the sack and the other surrounding tissues, the number of takes has been smaller. (3) The technique of injection. We find that the technique of injection has been of great importance. When the material was prepared as described above, the syphilitic testicle macerated thoroughly in a masticator, and often squeezed through cheese-cloth after that to remove tissue particles, and then this relatively clean material carefully injected into the center of the testicle itself, the percentage of takes has been much larger than in cases where the original material consisted of chancres or small indurations, where a reasonable amount of material could be obtained only with tissue detritus.

It seems to us also that some of the irregularity, especially in the early part of our work, may have been due to the fact that we have learned in the course of our studies that great frequency of examination is necessary in order that none of the lesions may be missed. Within the last month we have seen large, diffuse lesions which within 3 or 4 days have diminished in size, changing into small nodules, and in the course of another week have seemed to undergo complete retrogression. Rabbit 2 is an example of this. In this animal a large, diffuse lesion has completely disappeared within the course of a single week. Such rapid retrogression, of course, is noticed only in the cases of large, soft, myxomatous lesions, where there is no hard,

compact infiltration. The nodular and ulcerated lesions do not, of course, manifest the same speed of healing.

Other factors which must be considered in all such statistical studies are the frequent secondary infection or rapid necrosis of lesions and the almost invariable secondary infection of ulcerated lesions, in which even when the original focus was syphilitic, puncture reveals pus and bacteria, and only repeated puncture, and this with the chances against the investigator, may show treponemata under the dark-field.

Statements as to the difference in incubation time possessed by various strains have also engaged our attention, and we have been able to determine no regularity in this regard. In the case of the strains studied by us the incubation times have varied from 14 to over 190 days, and have averaged from 21 to 42 days. In the same strain large fluctuations have appeared, and we believe that this is subject to the modifying factors introduced by the nature of the material and manner of injection on the one hand, and the size and individual resistance of the rabbit injected, on the other. It does not appear from any of our observations that the length of incubation time has any relation at all to special characteristics of an individual strain. The incubation times observed by us were as follows:

Strain A	14	to	48	days.
" F	14	"	71	"
" L	14	"	192	"
" S	8	"	49	"
" T	14	"	74	"

In explanation of the table it should be said that two of the 14 day early lesions were proved by puncture, and that as regards the longest periods tabulated it may well have been that a more frequent examination of the rabbits might have shortened this possibly by as much as 5 days or a week. However, even subtracting such periods, the great length of incubation time in some cases is apparent.

An observation that has been emphasized, especially by Nichols, is the apparent similarity of lesions caused by the same strain, on the basis of which he believes that the human disease may be to a certain extent governed by the type of treponema with which the individual is infected. Although in our earlier studies we did not follow this

point carefully, during the later months of our work we have paid attention to it, and it does not appear that any of our strains, even the one sent to us by Dr. Nichols, has manifested such regularity in pathogenic powers. As illustrative examples we may cite a few only which we think are alone sufficient to show that, in our hands at least, the difference in lesion has been governed by other fortuitous factors rather than by any consistent and inherent properties of the particular treponema used. At the time of writing we have on hand the following lesions:

Strain A.

Rabbit 3. Small nodular lesions in both testicles.
" 4. A hard nodule in one testicle which has followed a large, diffuse lesion in the same testicle.
" 5. A chancre with beginning abscess formation.
" 6. A diffuse, gummy lesion in the left testicle.

Strain F.

" 7. A very small, superficial skin induration and a small nodule in the right testicle. Has had a small nodular lesion in the left.
" 8. A very small central nodule in one testicle.
" 9. A large, diffuse lesion in the right testicle, just beginning to become indurated in the center.

Strain S.

" 10. Two nodules, hard and indurated, one in each testicle at the lower pole. 2 weeks ago this rabbit had had diffuse lesions of the edematous or myxomatous variety.
" 11. Large, diffuse lesions 2 weeks ago which now show as ulcerated nodules or chancres.
" 12. A beginning small, hard nodule in one testicle.
" 13. Soft, general enlargement of the testicle which looks like an early diffuse lesion.

In Strain T, the strain sent us by Dr. Nichols, we have not been able to observe a uniformity of lesions, this strain apparently having changed either because of our technique or by reason of its prolonged rabbit passage. In this strain we have had, it is true, a preponderance of indurated plaques and nodules in the testicle, but in a number of animals, large, diffuse lesions and chancrous lesions have appeared. For instance, in our third and fourth generation of T, we have had the following lesions:

Strain T.

Rabbit 14. Developed a chancre.
" 15. Had an indurated plaque on the left testicle.
" 16. Had a small gelatinous nodule at the globotesticular junction with an edematous or myxomatous appearance of the whole testicle, which was loaded with treponemata. This testicle on palpation appeared to be normal, though generally enlarged.
" 17. Showed large, diffuse lesions of the myxomatous, soft type.
" 18. Another rabbit of the same generation of this strain as Rabbit 17 showed a nodule in the left testicle.

Neither in percentage of takes nor in speed of incubation time has this strain been markedly different from any of our others. The incubation time has varied from 14 to 74 days, which is well within the ranges shown by the others.

Another important point made by previous writers has been studied by us; namely, the relation of difference in morphology to pathogenicity. A distinct difference in thickness, as stated above, has been noted by so authoritative a writer as Noguchi, and we have looked for this in the rabbit *pallida*, often feeling sure that some of the strains did appear more slender and active than others. We have, however, taken lesions of four different strains and examined them with this particular point in view, having three observers check each other on the observation of the slides, without being able definitely to determine consistent difference in size. It is unquestionable that occasionally a strain looks thicker than other strains when taken from the testicle, but we have also seen thick and thin forms together in the same preparation. We have not measured, and do not wish to speak finally of this point, except in so far as we cannot at present definitely confirm consistent differences in morphology between the rabbit strains in our possession. We do not believe that such difference in the morphology, if it exists (and even in Noguchi's hands the difference between the narrowest and the thickest is estimated as 0.1 of a micromillimeter), has a constant relation to difference in invasive property.

CONCLUSIONS.

1. As regards these strains, at least, there is no consistent change, either increase or decrease, of rabbit pathogenicity during progressive rabbit passage.

2. There is no difference in pathogenicity between these different strains, although some of them were isolated from condylomata, some from chancres, one from a mucous patch, and one by Nichols from the nervous system.

3. We do not believe that there is any consistency of difference in speed of incubation between these various strains.

4. We think that the nature of the lesion produced by any individual strain, and the incubation time as well, are dependent upon the fortuitous factors incident to the nature of the material used, the technique of injection, and the size of rabbit in which the injection is made.

As to the tendency to generalize possessed by one strain or another, we are not prepared to make a statement. We have not inspected as carefully as we might all our rabbits in regard to the more obscure syphilitic lesions such as small nodules on the eyebrows, nodules on the nasal bones, and internal lesions of the eye. As a matter of fact, we have noticed three cases of keratitis, probably syphilitic, and a number of preputial metastases, we have seen occasional nodules about the anus, and in one case a peritonitis in the fluid of which living treponemata were found on examination. There has not been in our series an extensive number of generalizations. We attribute this largely to the fact that all the studies here recorded were made on rabbits intratesticularly inoculated. It appears from the writings of other investigators that intravenous and intracardial injection of rabbits leads to a more extensive metastatic or general distribution. Two of the few rabbits inoculated intravenously showed testicular lesions. A series of rabbits intracerebrally inoculated through trephine openings showed nothing definite on autopsy, though one of them showed interference with reflexes and rigidity of the limbs for a time which seemed significant of pathological change. This rabbit, however, recovered entirely.

STUDIES ON TREPONEMA PALLIDUM AND SYPHILIS.

IV. THE DIFFERENCE IN BEHAVIOR IN IMMUNE SERUM BETWEEN CULTIVATED NON-VIRULENT TREPONEMA PALLIDUM AND VIRULENT TREPONEMATA FROM LESIONS.

BY HANS ZINSSER, M.D., J. G. HOPKINS, M.D., AND MALCOLM McBURNEY, M.D.

(*From the Department of Bacteriology of the College of Physicians and Surgeons, Columbia University, New York.*)

(Received for publication, December 30, 1915.)

I. Agglutination.

In a paper published by two of the writers[1] in June, 1915, it was shown that, as other workers, notably Kolmer,[2] had found before, the inoculation of rabbits with culture *pallida* gives rise to the formation of powerful agglutinating properties in the sera of the animals. Definite agglutination was observed in dilutions of 1: 4,000, and the serum agglutinated not only the strain with which the rabbit had been immunized, but other strains, such as those cultivated by Noguchi. On the specific relationship of these agglutinins to treponemata other than *pallida* we shall report in another paper. The discovery of specific antibodies against the *pallida* in treated animals, of course, again gave us hope that it might be possible to determine whether or not the patient afflicted with active syphilis produced antibodies against the organism, a fact which has been much discussed and written about, but which up to the present time is uncertain. It is not our intention in this communication to review the literature of antibody formation in syphilis, which is extensive though disappointing as to results. We leave this for another paper in which we shall report observations on the presence of agglutinins for culture treponemata in the sera of human beings in the various stages of syphilis.

[1] Zinsser, H., and Hopkins, J. G., *Jour. Exper. Med.*, 1915, xxi, 576.
[2] Kolmer, J. A., *Jour. Exper. Med.*, 1913, xviii, 18.

In this paper we wish to call attention to what seems to be to us a fundamentally important fact; namely, the existence of a definite difference between the treponemata artificially cultivated and those derived directly from the lesions of infected man and animals.

In previous studies carried on in the course of the last two years, we have frequently looked for changes in motility and for agglutination of treponemata found in exudates from syphilitic lesions to which the sera of immunized rabbits had been added. Our results on these occasions were as inconclusive as those of other writers.

Hoffmann and von Prowazek[3] reported in 1906 that the serum of syphilitics in the later stages of the disease produced a diminution of motility in *Treponema pallidum*, an observation which was confirmed by Zabolotny[4] soon afterwards. Contradictory results, however, were obtained by Landsteiner and Mucha, [5, 6] who were unable to observe either immobilization or agglutination in syphilitic serum. Uhlenhuth and Mulzer[7] never observed agglutination in the sera of animals treated with virulent treponemata. Neisser and Bruck,[8] summarizing the work done up to 1911, express themselves as uncertain whether or not agglutinins in the serum of syphilitics had really been proven up to that time, when, of course, work with pure cultures had not been possible. The recent work of Kissmeyer[9] deals with the agglutination of culture treponemata in normal and syphilitic serum and will be left for consideration in a later paper of our own dealing with the same subject.

It is plain, therefore, that in the matter of agglutinin formation in the course of syphilis, nothing is yet definitely known, a state of affairs which unfortunately describes our knowledge of the general subject of antibody formation in this disease.

Having obtained powerful agglutinins in rabbits and sheep treated with culture treponemata, we undertook to determine the action of these sera upon the microorganisms obtained directly from lesions,

[3] Hoffmann, E., Cited from Neisser and Bruck, *Beitr. z. Path. u. Therap. d. Syph.*, Berlin, 1911, 205.

[4] Zabolotny, D., and Maslakowetz, *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1907, xliv, 532.

[5] Landsteiner, K., and Mucha, V., *Wien. klin. Wchnschr.*, 1906, xix, 1349.

[6] Landsteiner, K., and Mucha, V., *Centralbl. f. Bakteriol., 1te Abt., Ref.*, 1907, xxxix, 540.

[7] Uhlenhuth, P., and Mulzer, P., *Arb. a. d. k. Gsndhtsamte*, 1913, xliv, 307.

[8] Neisser, and Bruck, *Beitr. z. Path. u. Therap. d. Syph.*, Berlin, 1911, 205.

[9] Kissmeyer, A., *Deutsch. med. Wchnschr.*, 1915, xli, 306.

since to one habitually working with treponemata, a great many differences as to morphology, agility of motion, and primarily, of course, of virulence, between the culture organisms and the lesion treponemata are apparent. We therefore carried out a number of experiments, some of which are detailed in this paper, in which the action of serum was tested, in parallel series, upon both the culture organisms and those obtained from lesions.

After a considerable amount of experimentation it seemed to us that material obtained from human lesions, however good, or from rabbit lesions consisting of small nodules and chancres, was unsatisfactory in that the treponemata were so few in number and so intimately mingled with tissue detritus and blood derivatives that washing never resulted in a sufficient yield of microorganisms for satisfactory microscopic work. Incidentally, we may mention that in all agglutination work we have found it inaccurate to rely upon macroscopic results only; our readings had to be made both macroscopically and microscopically. We therefore chose as material only the large, diffuse, gelatinous lesions obtained frequently in rabbit testicles; and we used for most of our experiments such lesions obtained with our own Strain A, which had been cultivated and with which the immune serum had been produced. This gave us a strictly homologous experiment. In addition, we tested this serum on similar lesions obtained with our F and S strains.

In preparing material the testicles were removed and thoroughly macerated with a masticator. The macerated mass was then squeezed through cheese-cloth and, in favorable cases, a thick, gummy liquid was obtained which swarmed with treponemata, actively motile. Many lesions, of course, were worked over in this way without yielding favorable material, but in the experiments finally accepted only material was used in which a great many *pallida* were present relatively free from admixtures of foreign material. To the gummy fluid salt solution was added and the larger particles which had come through the cheese-cloth were thrown down by slow centrifugation. The *pallida* have apparently a low specific gravity and hardly any of the organisms are removed when centrifugation is neither at high speed nor prolonged. The supernatant fluid from this primary centrifugation was removed and allowed to settle in the

refrigerator over night. This removed more of the detritus. Dilution with salt solution was then practiced and very prolonged centrifugation, 3 to 4 hours at high speed, brought down most of the treponemata. These were washed in salt solution, and if considerable admixtures of tissue particles, etc., were still present, repetition of the process with more or less variation according to the nature of the material was practiced. When the final preparation was obtained, it usually consisted of about 0.5 to 1.0 cc. of a salt solution suspension, relatively clean of detritus, containing from four to six treponemata to the field, sometimes less, but in some cases considerably more. With material so prepared the experiments were set up.

EXPERIMENT I.

Agglutination of Virulent Treponemata with Serum of Rabbit 1.

Readings were taken macroscopically and microscopically after 2 hours at 37°C. and after 14 to 16 hours in the refrigerator.

The suspension of the Strain A culture material was made approximately equivalent to the virulent suspension.

Tubes set up.	Agglutination.
Strain A culture + Rabbit 1 serum, 1: 10.	+++
" " " + normal serum, 1: 10.	±
Virulent Strain A + Rabbit 1 serum, 1: 10.	±
" " " + normal serum, 1: 10.	±

EXPERIMENT II.

Comparative Agglutination of Virulent Strain A and Culture Strain A.

Very good, spongy lesion used; treated as described above. A suspension of Culture A was made which corresponded in number of treponemata to the virulent suspension obtained.

Serum of Rabbit 1 was used. This serum agglutinated Culture A in dilutions of 1: 2,000.

Tubes set up.		Agglutination.
1. Virulent Strain A	+ Rabbit 1 serum, concentrated.	±
2. " " "	+ " 1 " 1: 20.	–
3. " " "	+ salt solution.	–
4. Culture " "	+ Rabbit 1 serum, concentrated.	+++
5. " " "	+ " 1 " 1: 20.	++
6. " " "	+ salt solution.	–

EXPERIMENT III.

Strain S.

Suspension of Treponemata from a Gelatinous Lesion in a Rabbit Testicle.

Readings were made macroscopically and microscopically.

The suspension of Strain A culture material was made approximately equivalent to the virulent suspension.

Tubes set up.		Results.		
		1 hr. at 37° C.	2 hrs. at 37° C.	18 hrs. in ice chest.
1. Virulent Strain S	+ Rabbit 1 serum, 1:4.	0	0	0
2. " " "	+ normal rabbit serum, 1:4.	0	0	0
3. Culture Strain A	+ Rabbit 1 serum, 1:4.	+++	+++	+++
4. " " "	+ normal rabbit serum, 1:4.	±	±	++

EXPERIMENT IV.

Suspension of Virulent Strain S Used.

Readings were taken macroscopically and microscopically after 2 hours at 37°C. and after 14 to 16 hours in the refrigerator.

Tubes set up.		Agglutination.
Strain A culture	+ Strain A serum, 1:3.	+++
" " "	+ salt solution.	—
Virulent Strain F	+ Strain A serum, 1:3.	±
" " "	+ salt solution.	—

EXPERIMENT V.

Suspensions unfortunately very thin. Therefore Strain A culture suspension was made equally thin, about three or four microorganisms to the field.

The different columns represent readings by three different men in order to be sure that no individual error of judgment was possible.

Tubes set up.		Results.		
1. Virulent treponemata 3 drops	+ Rabbit 1 serum, 1:5, 3 drops.	—	—	—
2. Virulent treponemata 3 drops	+ Rabbit 1 serum, 1:20, 3 drops.	—	—	—
3. Virulent treponemata 3 drops	+ normal serum, 1:5, 3 drops.	—	—	—
4. Culture Strain A 3 drops	+ Rabbit 1 serum, 1:5, 3 drops.	++	++	++
5. Culture Strain A 3 drops	+ Rabbit 1 serum, 1:20, 3 drops.	++	++	++
6. Culture Strain A 3 drops	+ normal serum, 1:5, 3 drops.	+	++	++

These experiments show that an immune serum produced by the inoculation of rabbits with a culture of *Treponema pallidum*, although it contained powerful agglutinating properties for the culture *pallida*, had little or no effect upon the virulent *pallida* obtained directly from lesions. In Experiments II and IV there was slight but definite indication that a very weak effect had been produced upon the virulent treponema by the immune serum. We shall refer to this again, since it seems to indicate that if a sufficiently strong immune serum could eventually be produced, even the virulent *pallida* might be influenced. We need only refer to the long delayed success in passive immunization to the pneumococcus, where the capsulated nature of the organism rendered it unamenable to anything but the most powerful immune serum.

Furthermore, this apparent inability of the virulent *pallida* to go into relation with an antibody so powerfully active against the culture organism, going hand in hand with a loss in virulence on the part of the culture treponema, suggested the possibility of a change physiologically not unlike that associated in some of the bacteria with the acquisition and loss of a capsule.[10] Moreover, the difficulties of staining and general appearance variations of thickness and thinness observed in the *pallida* strains, together with the sticky, gelatinous nature of the lesions, also suggest to the observer the possibility that these organisms may either be capsulated or have a protoplasmic structure chemically not unlike that of the capsules of bacteria.

It occurred to us that we might be able to produce agglutinability in the virulent *pallida* by subjecting them to the acid and heat treatment introduced by Porges in experiments with the Friedländer bacillus group. Accordingly we carried out experiments in which virulent *pallida* material was treated with hydrochloric acid in a total concentration of 0.0625 N and heated in a water bath to from 70° to 80°C. for 15 minutes. The mixtures were made in very small agglutination tubes and measured with capillary pipettes by the Wright method, since, of course, it was difficult to obtain any quantities of the material for examination.

[10] Each of the writers individually has seen in virulent *pallida* suspensions occasional sheath-like structures which resemble capsules and which we have never seen in culture suspensions. We cannot yet stain these and the uncertainty of light effects in dark-field observations causes us to hesitate in making a positive statement.

EXPERIMENT VI.

Virulent Treponemata, Strain F.

Good lesion; treated as in preceding experiments.

Washed thoroughly in order to prevent the disturbing effects of possible coagulation of protein by the acid and heat treatment. Parts of the suspension were then made up to 0.0625 N hydrochloric acid and heated for 15 minutes at 80°C., and the volumes of the other fractions made equal with salt solution.

Tubes set up.	Result.	
1. Virulent Strain F, unheated, + salt (control).	+	Many free treponemata and few clumps of 3 to 8 individuals.
2. Virulent Strain F, unheated, + serum of Rabbit 1, 1:20.	++	Few free treponemata. Clumps larger than control.
3. Virulent Strain F, unheated, + serum of Rabbit 1, 1:50.	+	Perhaps slightly better clumping than control, but uncertain.
4. Virulent Strain F + acid + serum of Rabbit 1, 1:20.	+	Few treponemata caught in clumps of precipitate. Cannot make definite reading.
5. Virulent Strain F + acid + serum of Rabbit 1, 1:50.		Few treponemata caught in clumps of precipitate. Cannot make definite reading.
6. Culture Strain A + serum of Rabbit 1, 1:20.	++	
7. Culture Strain A + serum of Rabbit 1, 1:50.	++++	

EXPERIMENT VII.

Suspension made from material from large, diffuse lesion, Strain A, by the technique described above. Unusually good suspension obtained.

The following tubes were set up:

1. Suspension in 0.0625 N hydrochloric acid. } Heated at 75°C. for 15 minutes.
2. Suspension in salt solution. } Heated at 75°C. for 15 minutes.
3. Suspension in salt solution.

After two of the tubes had been heated for 15 minutes as above, Immune Serum 1 was added in concentration of 1: 25 to all three of the tubes.

No agglutination was observed in any of them after 48 hours' observation, macroscopically and microscopically.

The acid and heat experiments detailed above (Experiments VI and VII) necessarily consumed much time, and had to be repeated a number of times before we felt sure of the results. We are confident after critical repetition, however, that heating with acid, which according to Porges is sufficient to render inagglutinable capsulated bacteria agglutinable, does not have the same effect upon *Treponema pallidum.*

II. Treponemicidal Action.

In a preceding paper two of the writers have shown that the sera of rabbits and sheep immunized with culture *pallida* possess treponemicidal action upon culture *pallida* in dilutions higher than those possessing similar properties in the case of normal sera of rabbits and sheep. The experiences detailed above which indicated differences between the cultivated microorganisms and the virulent ones obtained from lesions in respect to agglutinability, led us to investigate also the treponema-killing properties of culture immune rabbit serum for virulent *Treponema pallidum.* Accordingly, taking serum from treated rabbits we allowed this serum to act upon treated organisms of varying kinds before injection into normal rabbit testicles. These experiments were as follows (Experiment I).

EXPERIMENT I.

Material from a lesion in Rabbit 19, A XIV. The testicular mass was macerated, the coarser particles thrown down in the centrifuge, and the supernatant fluid, which contained many motile treponemata, was divided into three parts, as follows:

I. 2.5 cc. virulent suspension + 5 cc. immune serum of Rabbit 20 (freshly taken and active).
II. 2.5 cc. virulent suspension + 5 cc. normal rabbit serum.
III. 2.5 cc. virulent suspension + 5 cc. salt solution.

These mixtures were allowed to stand for 1 hour at 37.5°C. There was no loss of motility or clumping in any of them. After 1½ hours it seemed that there were somewhat more non-motile forms in I than in the others. This appears to have been probably a deception. After 2 hours injections were made as follows:

0.75 Cc. Injected into Each Testicle.

I.	Rabbit	21.	Died after 26 days. Negative.
	"	22.	" " 26 " Positive lesion.
	"	23.	Killed for transfers after 40 days. Positive lesion.
	"	24.	Negative.
II.	"	25.	Positive.
	"	26.	"
	"	27.	Negative.
	"	28.	Positive.
III.	"	29.	Died after 26 days. Negative.
	"	30.	Positive.
	"	31.	Negative.
	"	32.	"

In this experiment it is plain that there was no protective power exerted by the action of the serum on the microorganism.

EXPERIMENT II.

Experiment carried out as before, except that no salt solution control was made. The suspension of virulent treponemata was divided into two parts, as follows:

I. Immune serum of Rabbit 33	5 cc.+	virulent suspension	2.2 cc.
II. Normal "	5 " +	" "	2.2 "

0.8 Cc. Injected into Each Testicle.

I.	Rabbit	34.	Negative.
	"	35.	"
	"	36.	Died before of value.
	"	37.	Positive.
II.	"	38.	Negative.
	"	39.	"
	"	40.	"

Experiment II, also, was entirely negative.

Since we had found in our treponemicidal experiments carried out upon culture organisms that the serum, just as in the case of bactericidal reactions, could be inactivated and reactivated, it seemed to us that insufficiency of alexin or complement might have been a source of error in our previous experiments. Therefore Experiment III was done.

EXPERIMENT III.

Virulent material suspended as before. The material was then divided into three parts and the following mixtures were made:

I.

	cc.
Virulent suspension	1.5
Immune serum, active	3.0
Normal rabbit serum	1.0

II.

Virulent suspension	1.5
Immune serum, active (dilution 1:20)	3.0
Normal serum	1.0

III.

Virulent suspension	1.5
Normal serum, active	4.0

These mixtures were incubated for 2 hours at 37.5°C. At the end of this time rabbits were inoculated in both testicles as follows:

1.0 Cc. Injected into Each Rabbit.

I.	Rabbit	41.	Negative.
	"	42.	"
	"	43.	Positive.
	"	44.	Negative.
II.	"	45.	"
	"	46.	Positive.
	"	47.	Doubtful.
	"	6.	Positive.
III.	"	48.	Negative.
	"	49.	Positive.
	"	50.	"
	"	51.	Negative.

These experiments as far as they have gone show no definite action of the immune serum upon the virulent treponemata, a result which is analogous to the agglutination experiments described in the first part of this paper.

It might be added that the technique by which these experiments had to be done still leaves considerable possibility of error. It has been impossible to obtain the virulent material free from a certain amount of tissue detritus, and dissolved protein which might in some way neutralize the action of antibodies in the immune serum. Fur-

thermore, it is possible that the serum may have definite action and yet the number of treponema placed in the mixtures in each case might have exceeded the maximum quantity that could be taken care of by the amounts of serum used, since, of course, it is well known to one who has carried out *in vitro* bactericidal experiments, that an excessive proportion of microorganisms will result in completely using up the immune serum, leaving unkilled bacteria in sufficient number to cloud by their growth all the bactericidal action. However, this latter possibility is, we think, rendered more or less unlikely by the last experiment where relatively large amounts of serum were used. We consider that these experiments justify the following conclusions, which to us indicate an important principle in questions of immunity in syphilis.

CONCLUSIONS.

Although antibodies can be produced by the immunization of animals with cultivated *Treponema pallidum*, and although these antibodies exert specific agglutinative and treponemicidal action upon the culture organisms, they possess, at least in the concentration so far obtained by us in rabbits and sheep, practically no action for virulent treponemata obtained directly from lesions. There seems to be in the infected body an inability to exert a purely serum action upon the virulent treponemata, a condition of affairs which may well lead to a lack of antigen absorption on the part of the body and a consequent failure to produce serum antibodies.

We do not think that this should in any way discourage our further investigation of the protective action of antibodies produced with culture *pallida*. On the one hand, the slight occasional agglutination and the lower proportion of takes with the concentrated serum in the last experiment at least indicate the possibility that we have been working with sera that are not sufficiently powerful and that just as with work with the pneumococcus and other highly invasive organisms, a serum of considerable antibody contents must be used before results can be expected. Again, the destruction of treponemata and the healing of lesions which undoubtedly takes place in rabbits, sometimes with surprising speed, may be a cellular destruction, and by injecting the sera either locally or intravenously and giving them time

to be absorbed by the cells before injecting virulent material, better results may be obtained. This direction of research as well as further studies on the antagonistic cellular processes against the *pallida*, the immunization of animals with killed virulent organisms, and the antibodies in rabbits and human beings during the course of infection and after recovery are being investigated, and we hope to be able to report upon them in the near future.

AN EXPERIMENTAL STUDY OF PAROTITIS (MUMPS).

BY MARTHA WOLLSTEIN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 1, 1915.)

PLATES 53 TO 55.

Studies dealing with the etiology of mumps have been comparatively few in number, and fall into two groups. In the earlier investigations, made chiefly in France,[1] but also in Germany[2] and in America[3] ordinary bacteriological methods were employed, and cocci were isolated from the blood, saliva, and fluid aspirated from the swollen parotid glands.

Attempts to produce parotitis in animals by the inoculation of these cocci proved entirely unsuccessful and the negative results of Roux and Pasteur,[4] and Fichera,[5] who failed to find the cocci demonstrated by other observers, further show that these early studies did not solve the problem of the etiology of epidemic parotitis. The studies made since 1908, when Granata[6] attacked the subject from a new point of view by using the filtrate of patients' saliva for animal inoculation, laid more stress on the reproduction of the disease in laboratory animals than on the isolation of a bacterium from human cases. Granata was the first to conclude that the virus of parotitis may be filterable, basing his conclusions upon the results obtained by inoculating the sterile saliva filtrate from two patients into the

[1] Charrin and Capitan, cited by Védrènes, *Mem. de méd. de chir. et de pharm. mil.*, 1882, xxxviii, 167. Laveran and Catrin, *Compt. rend. Soc. de biol.*, 1893, v, 528. Busquet, *Rev. de méd.*, 1896, xvi, 744. Tessier, P., and Esmein, C., *Compt. rend. Soc. de biol.*, 1906, lviii, pt. 1, 803, 853.

[2] Bien and Michaelis, M., *Verhandl. d. XV Cong. f. inn. Med.*, 1897, xv, 441.

[3] Mecray and Walsh, *Med. Rec.*, 1896, l, 440.

[4] Roux and Pasteur, cited by Védrènes, *Mem. de méd. de chir. et de pharm. mil.*, 1882, xxxviii, 167.

[5] Fichera, G., *Bull. d. r. Accad. di Roma*, 1905. xxxi, 29.

[6] Granata, S., *Med. ital.*, 1908, vi, 647, 672.

blood, parotid gland, and subcutaneous tissue of rabbits. A rise of temperature lasting three days followed the intravenous injections, and swelling of the parotid gland one to two weeks in duration resulted from the other inoculations.

Gordon[7] also used a filtrate of the saliva for the intracerebral inoculation of monkeys. Four of the animals died, having developed meningeal symptoms on the fourth day. At autopsy a lymphocytic meningitis was found with marked degenerative changes in the neurons of the cerebral cortex and anterior horns of the cord. Cultures from the meninges remained sterile. One monkey, inoculated intraperitoneally and intravenously, became ill on the eleventh day and showed swelling of the parotids and stiffness of both jaws, but without nervous symptoms. Recovery was complete. Attempts to transfer the "disease" from one monkey to another with filtrates of the brain and cord from the fatal cases proved entirely unsuccessful. The monkey which survived the longest showed an interstitial parotitis and an interstitial hepatitis, as well. Gordon concludes that mumps is due to a filterable virus of comparatively low virulence.

The work of Nicolle and Conseil[8] is interesting because of a mononuclear leukocytosis detected in one of three monkeys into whose parotid glands they had injected fluid aspirated from the parotid glands of children ill with mumps. After an incubation stage of sixteen to twenty-six days, the animals developed fever lasting two to seven days, and the parotid became swollen in one monkey. No bacteria were demonstrable in the fluid obtained from the human parotid glands.

It will be seen that the experiments of Granata and of Nicolle and Conseil give only suggestive, not entirely successful results so far as the reproduction of mumps in animals is concerned, while Gordon's intracerebral inoculations are chiefly interesting in view of the nervous symptoms sometimes exhibited by patients suffering from epidemic parotitis.

[7] Gordon, M. H., *Reports to the Local Government Board on Public Health and Medical Subjects*, London, 1914, N. S., No. 96.

[8] Nicolle, C., and Conseil, E., *Compt. rend. Acad. d. sc.*, 1913, clvii, 340.

EXPERIMENTAL PART.

Intratesticular inoculation of laboratory animals with infected materials has proved an aid to bacteriological research. The testis acts as an enriching medium, making cultural studies more possible. In addition, the pathological changes caused by the growth of the inoculated virus and the clinical symptoms resulting from such changes can be observed. The fact that orchitis is a frequent complication of mumps in human beings suggested that the animals used in an experimental study of the disease should be inoculated into the testis as well as into the parotid. Cultures from the inoculated organs could then be made according to Noguchi's anaerobic method, while the direct results of the inoculations could be studied. The latter only will be given in this paper.

Cats, rabbits, and monkeys were employed, but as neither rabbits nor monkeys gave promising results, the work was for the time being continued with cats alone.

We are indebted to Dr. Leopold F. Haas and Miss Gillaume, through whose courtesy material for study was obtained from children at the Home for the Friendless; to Dr. Alfred F. Hess and Dr. Sophie Rabinoff, who placed material at the Hebrew Infant Asylum at our disposal; and also to Dr. Matthias Nicoll, Jr., who sent us cases from the Department of Health of the City of New York. The method of obtaining material was uniform. Patients with swollen parotids and symptoms of one to three days' duration were asked to rinse the mouth with sterile salt solution and expectorate into a sterile glass dish. Whenever advisable the parotids were gently massaged at the same time. The secretion thus obtained was filtered through a new Berkefeld candle, and the clear filtrate tested for sterility by aerobic and anaerobic methods. It was found to be uniformly sterile.

The cats were anesthetized with ether, and the skin over the parotid region denuded of hair, then carefully cleansed. With a new, sharp needle, it was not difficult to enter the parotid gland and inject 1 cc. of fluid. Very large, old cats were not favorable because they have a thick layer of fat and dense connective tissue over the gland, making it difficult of access. The fluid is more apt to be injected into

this tissue than into the parotid and the resulting periglandular swelling obscures the results. Well grown young cats proved suitable. The testicles were prepared in the same way as the parotid, and no difficulty was encountered in injecting 1 cc. of fluid.

Before anesthesia the temperature of the cats was recorded, and an actual as well as a differential leukocyte count made. These tests were repeated daily throughout the period of observation.

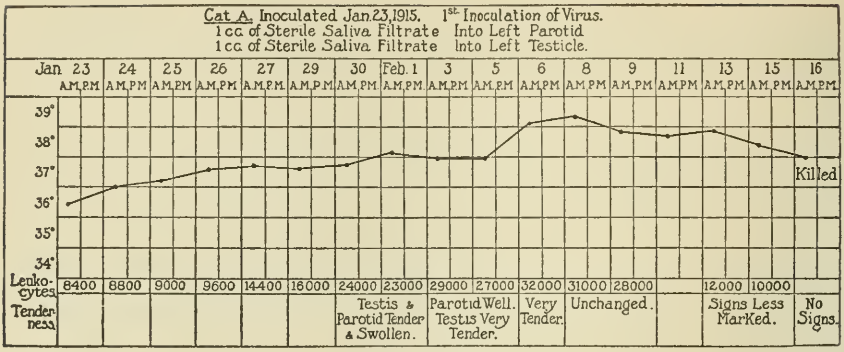
The animals recovered rapidly from the ether and showed no ill effects from the inoculations. The following day the temperature had usually risen 0.5° C., but the white blood cells had not increased in number. There was, as a rule, slight tenderness in the inoculated testicle, and often in the parotid as well. This was evidently of mechanical and not of inflammatory origin, and always disappeared within another twenty-four hours, leaving the cats apparently well on the second day, though the temperature was 0.5 to 0.8° C. above normal. After six or seven days, tenderness returned in the testis, accompanied by swelling; and similar symptoms appeared in the parotid. An increase in the leukocytes became apparent two days after inoculation, and reached the maximum in about seven to fourteen days, coinciding with the height of the fever. The swelling and pain in the parotid lasted two to five days, but the testicular swelling rarely subsided in less than ten to fourteen days. In the third week all the symptoms began to disappear, the leukocytes reaching the normal first, the tenderness disappearing at the same time, and the fever persisting for another week. While tenderness on palpation of the parotids was less marked than that of the testes, and the swelling never reached the stage of marked facial asymmetry, the cats nevertheless manifested some degree of discomfort in the inoculated parotid. The appetite was only slightly affected, and at no period of the experiments did the cats seem especially ill. The disease was not fatal in any instance. The following protocol is typical.

Cat A.—Inoculated Jan. 23, 1915, with sterile filtrate made from the saliva of four children who had been ill two days. 1 cc. injected into left parotid and into left testicle, respectively (Protocol I, Text-fig. 1).

PROTOCOL I.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°*C.*		
Jan. 23	36.4	8,400	Polynuclears 60; large mononuclears 19; small mononuclears 20; eosinophils 2.
" 24	37.0	8,800	Animal seems well. Left testis slightly tender.
" 25	37.2	9,000	No signs.
" 26	37.6	....	
" 27	37.7	9,600	No signs. Polynuclears 71; large mononuclears 14; small mononuclears 14; eosinophils 1.
" 29	37.6	14,400	No signs. Polynuclears 73; large mononuclears 14; small mononuclears 13; eosinophils 3.
" 30	37.8	24,000	Left testis quite tender and slightly swollen. Parotid tender. Polynuclears 70; large mononuclears 14; small mononuclears 13; eosinophils 1.
Feb. 1	38.2	23,000	Polynuclears 75; large mononuclears 8; small mononuclears 16; eosinophils 1. Signs unchanged.
" 3	38.0	29,000	Polynuclears 64; large mononuclears 16; small mononuclears 19; eosinophils 1. Left testis very sensitive and swollen. Parotid signs have disappeared.
" 5	38.0	27,000	Signs the same.
" 6	39.2	32,000	Polynuclears 64; large mononuclears 13; small mononuclears 22; eosinophils 1. Tenderness very marked.
" 8	39.4	31,000	No change.
" 9	38.8	28,000	" "
" 11	38.7	....	
" 13	38.9	12,000	Tenderness less marked.
" 15	38.5	10,000	No tenderness. Left testis slightly smaller than right.
" 16	38.0	....	Chloroformed.

Left testis smaller; on section it is grey and cloudy, but scarcely firmer than the right; neither hemorrhages nor other lesions apparent. Left parotid no larger than right; on section nothing obvious. Microscopically foci of cellular infiltration are found in both parotid and testis, and epithelial degeneration in the latter. The uninoculated glands on the right side are quite normal.



Text-Fig. 1.

Transmission from Cat to Cat.

At intervals varying from seven days to four weeks, the animals were etherized, the inoculated glands removed with aseptic precautions, and extracts or emulsions from them injected into other cats. The extracts were prepared in the following way: The organs were cut into small pieces, thoroughly ground up in a mortar with sterile sand and salt solution in a proportion of about 1 to 10, shaken for 2 to 3 hours at 37° C., and centrifugalized. The opalescent fluid was used for injection after it had been tested for sterility. Emulsions were made by grinding the organs in a small tissue-grinding machine through whose fine wire screen 0.2 to 0.3 cc. of finely divided material passed. This was suspended in 1 to 2 cc. of salt solution and its sterility tested before use. The best time for the reinoculation was found to be from the fourteenth to the seventeenth day when the testicular swelling, the leukocytosis, and the fever were highest. During the first seven to ten days transfers were less uniformly successful. Evidently the reaction requires about two weeks for its maximum development. Atrophy of the inoculated testicle occurred in several cats, after the acute symptoms had abated.

After the third or fourth transfers the reaction developed more rapidly and the effects were more severe. But after the sixth or seventh transfers the reaction perceptibly declined. In one instance only were effects observed at the eighth transfer.

Certain illustrative experiments are given in the text-figures and protocols which follow.

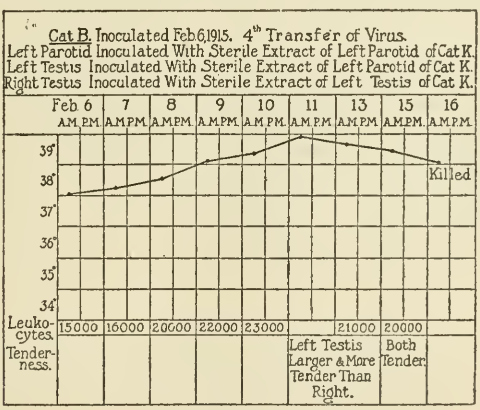
Cat B.—Fourth transfer of Strain III. Inoculated Feb. 6, 1915, with sterile extracts of parotid and testis from Cat K (Protocol II, Text-fig. 2).

Left parotid injected with sterile extract from left parotid.
“ testis “ “ “ “ “ “ “
Right “ “ “ “ “ “ “ testis.

PROTOCOL II.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°*C.*		
Feb. 6	38.0	15,000	Polynuclears 62; large mononuclears 17; small mononuclears 19; eosinophils 2. No signs.
“ 7	38.3	16,000	
“ 8	38.5	20,000	Polynuclears 70; large mononuclears 16; small mononuclears 16; eosinophils 4. No signs.
“ 9	39.1	....	
“ 10	39.8	22,000	
“ 11	39.9	23,000	Polynuclears 76; large mononuclears 11; small mononuclears 11; eosinophils 2. Left testis tender; right less so.
“ 13	39.7	21,000	Polynuclears 75; large mononuclears 13; small mononuclears 8; eosinophils 4. Left testis very tender and enlarged.
“ 15	39.5	20,000	
“ 16	39.0	....	Chloroformed.

Left testis larger than right; on section both are cloudy and moist compared with those from a normal cat. The left parotid measures 3.5 × 2.5 cm., while the right is 3 × 2.5 cm. in diameter. The left is pinkish in color and more moist and granular on section. Microscopically the right testis shows a more marked degree of "spermatorrhexis" than the left.



TEXT-FIG. 2.

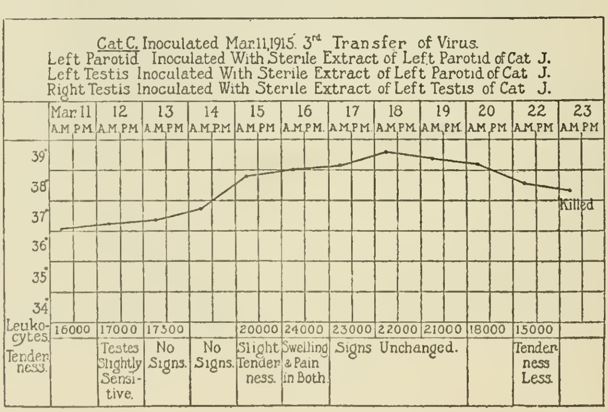
Cat C.—Third transfer of Strain IV. Inoculated Mar. 11, 1915, with sterile extracts of parotid and testis from Cat J (Protocol III, Text-fig. 3).

Left parotid injected with extract from left parotid.
" testis " " " " " "
Right " " " " " " testis.

PROTOCOL III.

Date.	Tempera-ture.	Leukocytes.	Remarks.
1915	*°C.*		
Mar. 11	37.0	16,000	Polynuclears 62; large mononuclears 16, small mononuclears 18; eosinophils 4.
" 12	37.3	17,000	Testes slightly sensitive.
" 13	37.4	17,500	No signs.
" 14	37.8	....	" "
" 15	38.8	20,000	Very slight tenderness.
" 16	39.0	24,000	Polynuclears 77; large mononuclears 11; small mononuclears 9; eosinophils 3. Swelling and tenderness in both testes.
" 17	39.2	23,000	Signs the same. Parotid not sensitive.
" 18	39.6	22,000	Polynuclears 79; large mononuclears 6; small mononuclears 10; eosinophils 5. Signs unchanged.
" 19	39.4	21,000	
" 20	39.2	18,000	Polynuclears 70; large mononuclears 4; small mononuclears 18; eosinophils 8. Signs unchanged.
" 22	38.8	15,000	Tenderness less
" 23	38.6	....	Chloroformed.

Testes scarcely enlarged, but left one, on section, is firmer than the right; both are cloudy. Left parotid is pinkish in color but not larger than the right. Microscopically both testes show marked epithelial degeneration, and, in addition, the left is the seat of cellular infiltration (Fig. 3). Cultures sterile.



TEXT-FIG. 3.

Control Experiments with Normal Organs.

As control experiments, extracts were prepared from the parotid glands and testicles of normal cats, and injected into three healthy cats in doses equal to those previously used. Neither enlargement nor tenderness developed in the glands inoculated in this way nor did the rise in temperature last longer than two days. Coincident with the temperature rise there was a polynuclear leukocytosis of two days' duration. At autopsy no macroscopic or microscopic changes were demonstrable in either the parotids or the testicles, while active spermatogenesis was in progress.

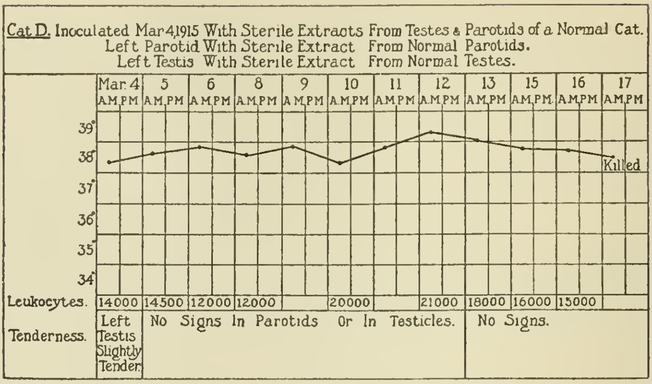
Cat D.—Inoculated Mar. 4, 1915, with sterile extract of parotid and testis from a normal cat (Protocol IV, Text-fig. 4).

Left parotid injected with sterile extract from left parotid.
" testis " " " " " " testis.

PROTOCOL IV.

Date.	Temperature.	Leukocytes.	Remarks.
1915	*°C.*		
Mar. 4	38.3	14,000	Polynuclears 65; large mononuclears 12; small mononuclears 20; eosinophils 3.
" 5	38.6	14,500	Left testis slightly tender.
" 6	38.8	....	No signs.
" 8	38.6	12,000	Polynuclears 69; large mononuclears 13; small mononuclears 7; eosinophils 1. No signs.
" 9	38.8	....	
" 10	38.4	20,000	Polynuclears 65; large mononuclears 10; small mononuclears 12; eosinophils 3. No signs.
" 11	38.9	....	
" 12	39.4	21,000	Polynuclears 84; large mononuclears 8; small mononuclears 4; eosinophils 4. No signs.
" 13	39.0	18,000	Polynuclears 71; large mononuclears 20; small mononuclears 6; eosinophils 3. No signs.
" 15	38.8	16,000	Polynuclears 64; large mononuclears 28; small mononuclears 6; eosinophils 2. No signs.
" 16	38.8	15,000	Polynuclears 66; large mononuclears 14; small mononuclears 18; eosinophils 2. No signs.
" 17	38.5	....	Chloroformed.

No difference observed in the parotids and testes on the two sides, on macroscopic and on microscopic examination. Spermatogenesis appeared normal.



Text-Fig. 4.

Control Experiments with Normal Saliva.

Other control experiments were made by injecting the sterile filtrates of normal saliva obtained from three healthy persons into the parotid glands and testes of normal cats. The resulting rise in temperature was not greater than 0.5° C. While the leukocytes were only increased by two or three thousand cells, the polymorphonuclear cells were relatively more numerous after the injection, and they did not regain the normal level during the period of observation which lasted from twelve to sixteen days. The small mononuclear cells were relatively diminished. Neither swelling nor tenderness developed in the inoculated glands, and even after the third and fourth successive transfer with this material no macroscopic lesions were discernable at autopsy. On microscopical examination the parotid glands showed no difference between the inoculated and the uninoculated side, and the same was true of the testes in seven of the animals. In two cats, however, the inoculated testis showed a moderate amount of degenerative change in the tubular epithelium. The lesion, however, was not comparable to that in the testes of the cats inoculated with saliva from mumps patients, being far less marked in extent and in degree. It should simply be mentioned that in two instances, the testes of cats into which saliva filtrates from healthy persons had been

injected were not absolutely normal on microscopic examination after death. Protocol V is typical.

Cat E.—Inoculated Oct. 8 with sterile filtrate of normal saliva into left testis and right parotid gland (Protocol V).

PROTOCOL V.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°*C.*		
Oct. 8	38.6	30,000	Polynuclears 50; large mononuclears 8; small mononuclears 35; eosinophils 7. Slight tenderness of left testis.
" 9	38.3	32,000	Polynuclears 67; large mononuclears 8; small mononuclears 15; eosinophils 10. No tenderness of testis or parotid.
" 11	38.7	33,000	Polynuclears 61; large mononuclears 7; small mononuclears 27; eosinophils 5.
" 13	39.0	29,000	Polynuclears 63; large mononuclears 7; small mononuclears 18; eosinophils 12.
" 14	38.8	32,000	Polynuclears 73; large mononuclears 3; small mononuclears 20; eosinophils 4.
" 15	39.0	30,000	Polynuclears 21; large mononuclears 5; small mononuclears 18; eosinophils 6.
" 16	38.8	25,000	Polynuclears 66; large mononuclears 4; small mononuclears 20; eosinophils 10.
" 18	38.5	22,000	Polynuclears 76; large mononuclears 5; small mononuclears 10; eosinophils 9.
" 19	39.0	32,000	Polynuclears 77; large mononuclears 4; small mononuclears 13; eosinophils 6.
" 20	39.0	30,000	Polynuclears 65; large mononuclears 10; small mononuclears 22; eosinophils 3. No tenderness.
" 21	38.2	....	Chloroformed.

At autopsy there was no difference in the size, color, or consistency of testes or parotid glands.

Microscopically no lesion was found in the inoculated testis or parotid.

Finally sterile salt solution alone was injected into the parotid gland and testes of three cats. The temperature rose only half a degree, and neither microscopic nor macroscopic lesions developed.

Pathology.

1. *Gross Appearance.* (*a*) *Parotid Gland.*—On exposing the parotid glands in the recently killed cats, the deeper pink color of the inoculated gland was very striking, as was also its larger size. The difference in weight varied from 70 to 340 mg. The inoculated gland was more moist than the uninoculated, and showed on section a granular appearance, due to swelling of the acini. No other changes occurred. The adjacent lymph nodes were usually congested but not distinctly enlarged (Fig. 5). The molar gland, situated just beneath the skin at the angle of the mouth and extending along the lower lip, was uniformly congested and swollen on the inoculated side. During life the buccal mucosa over the gland was distinctly reddened. This symptom could be found in the cat's mouth in four or five days after inoculation and in a few instances was accompanied by swelling. The opening of the duct leading from the injected parotid was always surrounded by a small zone of congestion.

(*b*) *Testis.*—The inoculated testis was larger than the uninoculated, but unchanged in color. On section the cut surface was more gray, cloudy, and moist. In a few instances the point of inoculation was visible as a small dark spot, but otherwise no focal changes were noted.

2. *Microscopic Appearance.* (*a*) *Parotid.*—The histological changes when present in the parotid were not constant for all the transfers of every strain. In the first transfer they were usually inconspicuous; and at most congestion of the vessels and swelling of the epithelium of the acini were made out, with edema of the interlobular connective tissue.

The most marked changes appeared coincidently with the third and fourth transfers. In some examples, the glands showed infiltration of the interlobular connective tissue with mononuclear and a few polynuclear cells in addition to the edema The infiltration was most intense about the secretory ducts (Fig. 1) which were sometimes dilated (Fig. 2). The epithelium of the acini was swollen and cloudy in these instances. The areas of cellular infiltration were more pronounced in some parts of the gland than in others, were always multiple, and could be easily differentiated from the small

lymph nodes which are normally present between the lobules. In some instances infiltration did not appear at all.

(*b*) *Testis*.—The histological changes in the testis were more constant than were those in the parotid, and, in further contrast, they were more pronounced in the epithelium than in the supporting framework of the gland. The layer of cells next to the basement membrane of the tubules tended to be normal in appearance, but the rest of the cells were often the seat of a change resulting in a diminution in the number of spermatocytes and consequently of the mitotic nuclei normally found. The spermatids were even more altered, showing as the remains of their nuclei irregular and deeply staining granules, while the cell bodies stained poorly or had undergone extensive lysis. In these cases, spermatozoa, as would be expected, were diminished in number and few were normal in appearance, their broken-up condition being easily discernible (Fig. 3). This process of imperfect spermatogenesis and of disintegration or spermatorrhexis was further indicated by the empty condition of the tubules of the rete and epididymis. Epithelial changes of some degree were present in some part of practically all the inoculated testicles. Interstitial cellular infiltration, on the other hand, occurred far less frequently in the testicle than in the parotid gland. In a few instances it was pronounced about groups of tubules, whose epithelium showed marked degeneration (Fig. 4). Another striking change in the testis was noted in the interstitial cells. These were larger than normal, and, in several instances, actually increased in number (Fig. 3), forming large masses between the tubules.

Fibrous connective tissue in increased amount with a diminution in the size and number of the convoluted tubules was found in the atrophic testes.

The microscopic examinations were always made with material removed from animals killed with chloroform and fixed at once in Zenker's fluid.

Reinoculation Experiments.

Four cats which reacted well were allowed to survive until all symptoms abated and were then reinjected with filtrates prepared from fresh cases of mumps in the manner already described (page

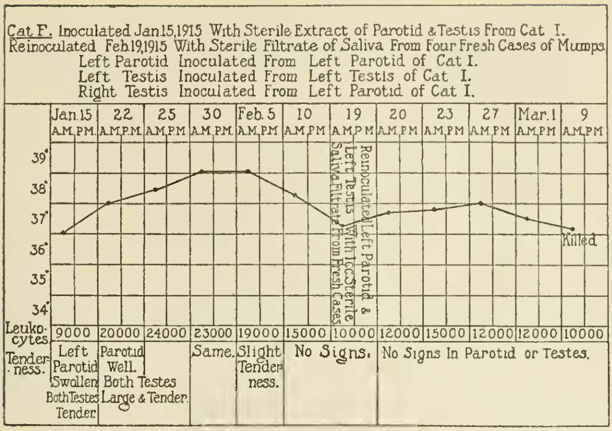
358). As a result of the second injection the temperature rose only 0.8° C., and the leukocytes increased only by 5,000 cells, whereas after the first injection the rise of temperature was about 1.9° C. above normal, and the white cells had more than doubled. No sensitiveness of either parotid or testis developed after the second injection. This result suggested that the first injection had reduced considerably the reaction to the second. Whether the effect was one of immunization can only be conjectured.

Cat F.—Inoculated Jan. 15, 1915, with sterile extract of parotid and testis from Cat I, making this cat the third transfer of this series (Protocol VI, Text-fig. 5).

Left parotid injected with left parotid of Cat I.
" testis " " " testis " " "
Right " " " " parotid " " "

PROTOCOL VI.

Date.	Temperature.	Leukocytes.	Remarks.
1915	*°C.*		
Jan. 15	37.0	9,000	Polynuclears 66; large mononuclears 17; small mononuclears 12; eosinophils 5.
" 22	38.0	20,000	Polynuclears 80; large mononuclears 15; small mononuclears 5; eosinophils 0. Left parotid tender and swollen; both testes tender.
" 25	38.4	24,000	Parotid swelling gone. Both testes tender and enlarged.
" 30	39.0	23,000	Signs unchanged.
Feb. 5	39.0	19,000	Tenderness of testes slight. Polynuclears 60; large mononuclears 14; small mononuclears 26; eosinophils 0.
" 10	38.3	15,000	No signs. Polynuclears 62; large mononuclears 15; small mononuclears 20; eosinophils 3.
" 19	37.2	10,000	No signs. Reinoculated into left testis and left parotid with 1 cc. of sterile filtrate from 4 fresh cases of mumps.
" 20	37.6	12,000	Polynuclears 68; large mononuclears 15; small mononuclears 15; eosinophils 2. No signs.
" 23	37.8	15,000	Polynuclears 72; large mononuclears 12; small mononuclears 13; eosinophils 3.
" 27	38.0	12,000	Polynuclears 70; large mononuclears 13; small mononuclears 15; eosinophils 2. No signs.
Mar. 1	37.5	12,000	No signs.
" 9	37.2	10,000	" " Chloroformed.



TEXT-FIG. 5.

Serum Protection Experiments.

In order to test the protective power of the serum of a cat which had entirely recovered from an inoculation with the filtrate three months before, three experiments were made. Material from the enlarged glands of cats killed at the height of the reaction had been kept in glycerine in the ice box for four months. This was emulsified in the usual way and the emulsion was left in contact with the serum for two hours at a temperature of 37° C. The mixture was then injected into the parotid gland and testicle of normal cats, control animals being inoculated at the same time with glycerinated material which had not been treated with the serum. A definite difference in the effects was noted in the two sets of animals. The animals receiving the emulsion-serum mixture showed less reaction than those receiving the emulsion alone. Glycerination of the testicles and parotids seemed to make little change, within the period of the experiment, in the results of the inoculation.

The protocols and text-figures illustrating the experiments follow.

1. Control.

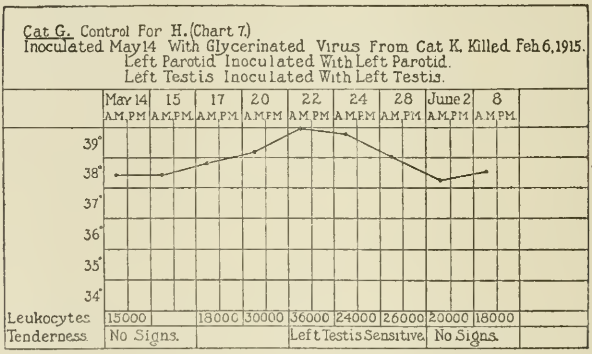
Cat G.—Inoculated May 14, 1915, with glycerinated material from Cat K, killed Feb. 6 (Protocol VII, Text-fig. 6).

Left parotid inoculated with left parotid of Cat K.
" testis " " " testis " " "

PROTOCOL VII.

Date.	Temperature.	Leukocytes.	Remarks.
1915	*°C.*		
May 14	38.4	15,000	Polynuclears 68; large mononuclears 10; small mononuclears 14; eosinophils 8.
" 15	38.4	....	No signs.
" 17	38.8	18,000	" " Polynuclears 78; large mononuclears 7; small mononuclears 12; eosinophils 3.
" 20	39.1	30,000	No signs. Polynuclears 70; large mononuclears 13; small mononuclears 8; eosinophils 9.
" 22	40.0	36,000	Left testis sensitive.
" 24	39.8	24,000	" " " Polynuclears 59; large mononuclears 14; small mononuclears 20; eosinophils 7.
" 28	39.0	26,000	Left testis sensitive. Polynuclears 65; large mononuclears 12; small mononuclears 17; eosinophils 6.
June 2	38.3	20,000	No signs. Polynuclears 67; large mononuclears 9; small mononuclears 21; eosinophils 3.
" 8	38.5	18,000	No signs.

Animal survived.



TEXT-FIG. 6.

2. Serum Experiment.

Cat H.—Inoculated May 14, 1915, with glycerinated material from Cat K, kept in contact with serum of Cat L for two hours at 37° C. (Protocol VIII, Text-fig. 7). Cat L was inoculated Feb. 6; well since Mar. 1; bled on May 12.

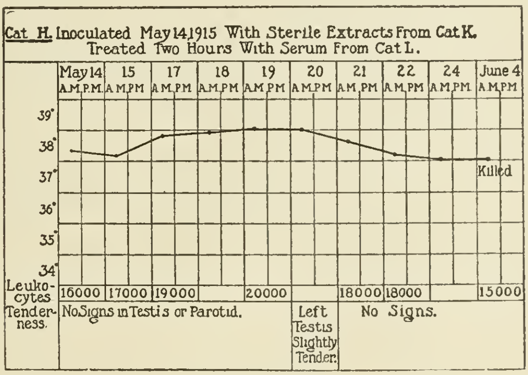
Left parotid inoculated with left parotid of Cat K.

" testis " " " testis " " "

PROTOCOL VIII.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°C.		
May 14	38.3	16,000	Polynuclears 70; large mononuclears 12; small mononuclears 12; eosinophils 4.
" 15	38.2	17,000	No signs.
" 17	38.9	19,000	Polynuclears 74; large mononuclears 10; small mononuclears 13; eosinophils 3. No signs.
" 18	38.9	....	
" 19	39.0	20,000	Polynuclears 69; large mononuclears 13; small mononuclears 14; eosinophils 4. No signs.
" 20	39.0	....	Left testis slightly tender.
" 21	38.6	18,000	No signs.
" 22	38.2	18,000	Polynuclears 71; large mononuclears 14; small mononuclears 13; eosinophils 2. No signs.
" 24	38.0	....	No signs.
June 4	38.0	15,000	" "

Chloroformed. Neither parotid nor testis showed any lesion on gross or microscopic examination.



TEXT-FIG. 7.

In comparing the case histories of the two cats, it will be noted that there are differences in the temperature and leukocyte counts, as well as in the reaction within the organs. Thus while the temperature of the control animal rose to 40° C., and remained above 39° for a week, the cat injected with the serum-treated material showed a rise to 39° C. over a period of only two days. Again, the

white cells of the control had increased to double their initial number on the eighth day after inoculation, and had not returned to normal ten days later; in the serum cat, on the contrary, they had risen only 4,000 in actual number by the fifth day, and the count had become practically normal within a week. The differential count remained quite unchanged in the second animal, while the control showed the early characteristic polynuclear cytosis and the later lymphocytosis.

The serum from a normal cat which had not been inoculated was employed in like manner to the immune serum, to serve as a serum control. The normal serum did not inhibit the action of the virus, as Protocol IX indicates.

Cat M.—Inoculated Dec. 15 with sterile extract of parotid and testis of an inoculated cat, which had been kept in contact with normal cat serum for 2 hours at 37°C. Right parotid and left testis injected.

PROTOCOL IX.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°*C.*		
Dec. 15	38.9	21,000	Polynuclears 62; large mononuclears 19; small mononuclears 14; eosinophils 5.
" 16	38.9	23,000	No signs.
" 17	39.3	....	
" 18	39.2	25,000	Polynuclears 65; large mononuclears 21; small mononuclears 12; eosinophils 1.
" 20	39.3	30,000	Left testis tender.
" 21	39.2	....	" " more tender.
" 22	39.5	32,000	
" 23	39.7	....	" " tender.
" 24	39.6	35,000	" " "
			Polynuclears 56; large mononuclears 18; small mononuclears 21; eosinophils 5.
			Chloroformed.

At autopsy the right parotid was distinctly swollen, and more congested than the left. The left testis was slightly larger than the right.

These experiments indicate that the serum of the cats employed has the power to reduce the development of the reaction produced by the injection of testicular and parotid materials derived from cats treated with filtrates of the saliva from cases of acute parotitis.

Blood Changes.

Daily blood counts were made on several normal cats, to ascertain the blood picture in healthy animals. It was found that the average number of leukocytes varied from 16,000 to 20,000, and that a difference of 5,000 from day to day was not unusual. It follows, therefore, that only a very decided increase in the number of white cells can have any significance in the cat.

The white cells began to increase within the first forty-eight hours after the inoculations, while on the seventh or eighth day a sudden additional rise, often doubling the initial count, may occur. The increase is maintained, with daily fluctuations, for a period of eight to ten days, the maximum being reached on about the fourteenth to the sixteenth day. The fall in number takes place gradually, the initial number being reached as a rule in about four weeks. During the first two weeks the leukocytosis is polymorphonuclear in character. At the end of the second or beginning of the third week, when the fever and glandular swellings are at the highest, a lymphocytosis is noted. The large mononuclear and eosinophil cells remained practically unchanged throughout.

The blood picture as described in the experiments is not very dissimilar from the one observed in cases of parotitis in man with testicular complications. Wile[9] has noted the change from a lymphocytosis to a polynuclear leukocytosis under these conditions. Apparently the change is inconstant, since Feiling[10] has not always found it. Recently Aubertin and Chabanier[11] have noted an initial polynuclear leukocytosis in all cases of mumps examined, the lymphocytosis following later, a fact which does not agree with Feiling's observations, which are to the effect that a relative and absolute lymphocytosis is present on the first day of epidemic parotitis. The increase in mononuclear cells found by Nicolle and Conseil in one of their monkeys, which had been inoculated into the parotid but not into the testicle, is of some interest in this connection, as is the polynuclear leukocytosis without the later appearance of a lymphocytosis

[9] Wile, I. S., *Arch. Pediat.*, 1906, xxii, 669.

[10] Feiling, A., *Quart. Jour. Med.*, 1915, viii, 255.

[11] Aubertin, Ch., and Chabanier, H., *Arch. d. mal. du coeur*, 1915, viii, 1.

noted in cats inoculated intratesticularly with material from normal cats.

That the polynuclear leukocytosis is not due to suppurative inflammation of the testes is indicated by the microscopic examinations, which showed also that at the time the testicles were removed no excess of polynuclear cells occurred in them.

DISCUSSION.

The object of the study described in the preceding pages was the reproduction of the chief organic lesions of parotitis in animals by means of filtered extracts of the saliva derived from persons suffering from acute parotitis. The experiments indicate that certain definite results have been obtained. It now appears that the injections into cats of bacteria-free filtrates of the saliva derived from cases of acute parotitis are capable of setting up a series of pathological changes in the parotids and testicles, expressed by fever, leukocytosis, tenderness, and swelling attended by definite histological alterations. Whether these changes accurately reproduce the condition arising in man in acute parotitis need not now be decided. The interest of the observations increases, however, in view of the fact that the effect of the injections is intensified by successive transfers of the inoculated organs from animal to animal through several passages.

Upon what principle present in the saliva the effects produced depend need not be discussed at the moment. It is obvious that ordinary bacteria can be excluded, since they were never found in microscopic preparations of the filtrate, or in aerobic or anaerobic (tissue) cultures, or in film preparations and sections made from the inoculated parotids and testes. On the other hand, it seems necessary to suppose that the active agent is of living nature; and thus the conclusion of a minute filterable virus is suggested.

The effects attributed to the saliva from cases of parotitis do not follow the injection of filtrates prepared with the saliva of normal persons. Moreover, and this point is a suggestive one, the active agent present in the saliva in cases of parotitis is neutralized or rendered ineffective by the serum of a cat which had been permitted to survive the injection of testicular and parotid emulsions, while the serum of a

normal cat had no such power. Assuming the activity of the salivary filtrate to be due to a living virus the power of prevention exercised by the serum may be assumed to be due to an immune body which was developed in the inoculated cat.

SUMMARY AND CONCLUSION.

Cats injected into the parotid gland and testicle with a bacterial sterile filtrate of the salivary secretion of children in the active stage of parotitis or mumps can be made to develop a pathological condition having several points of resemblance to the condition present in mumps in human beings.

After an incubation stage of from five to eight days definite changes have been noted in the temperature, blood leukocytes, and inoculated organs.

The temperature rise begins within twenty-four hours of the inoculations and reaches a maximum in from seven to fourteen days. The febrile rise fluctuates between 1° and 2.5° C.

The white blood cells begin to increase on the second day following the inoculation. The first change is a polymorphonuclear leukocytosis which precedes the glandular swellings. This initial rise is followed by a decline, after which the lymphocytes increase. The increase is confined to the small lymphocytes, which increase to from 7 to 10 per cent of their initial number.

The inoculated glands become swollen and tender. The swelling and tenderness become apparent from the fifth to the ninth days and persist for a variable period. The parotid changes are less constant or less obvious than are the testicular. The latter are constant and endure from eight to twelve days.

The rise of temperature and the leukocytosis precede the glandular swelling, but all the changes reach the maximum at about the same time, after which they decline gradually. What may be regarded as normal conditions are reestablished in four weeks or less.

The intraparotid and intratesticular injections of extracts of normal parotid gland and testicles may cause a mild rise of temperature and leukocytosis of brief duration, but swelling and tenderness are absent. The white cells increased are the polymorphonuclears and not the lymphocytes.

The intraparotid and intratesticular injections of filtrates of normal saliva may cause a mild rise of temperature of very brief duration, but leukocytosis, swelling, and tenderness do not appear.

The histological changes in the parotid gland when present consist chiefly of edema of the interlobular connective tissue with mononuclear interstitial infiltration about the ducts and elsewhere. In cases of long duration the ducts may be dilated. But in some instances the swollen gland while showing congestion and edema in gross showed inconspicuous changes under the microscope. The histological changes in the testicle are of two kinds: inconstant changes of cellular invasion between the tubules and swelling or even multiplication of the interstitial cells, constant ones consisting of degeneration of the epithelium and interference with spermatogenesis, a condition to which we have applied the term "spermatorrhexis."

The pathological conditions set up by the filtrate derived from the salivary secretion of cases of acute parotitis are intensified by successive transfers through a small series of cats of the extract and emulsion of the parotid gland and testicle previously inoculated.

The pathological changes are also prevented or reduced when the extract or emulsion is previously incubated with a quantity of blood serum obtained from a cat which has survived inoculation. Normal serum, on the other hand, has no such inhibiting effect.

The deduction from these experiments is to the effect that the salivary secretion in parotitis or mumps contains a filterable substance capable of setting up a series of definite pathological conditions when inoculated into the testicle and parotid glands of cats. Whether this active material is a microorganism and if so whether it is the specific microbic cause of parotitis or mumps remains to be ascertained.

EXPLANATION OF PLATES.

Plate 53.

Fig. 1. Parotid gland, showing cellular infiltration around the secretory ducts. Cat killed after 18 days.

Fig. 2. Parotid gland, showing cellular infiltration and dilatation of the secretory ducts. Cat killed after 17 days.

Plate 54.

Fig. 3. Testicle, showing cellular infiltration around the tubules, and spermatorrhexis. Cat killed after 12 days.

Fig. 4. Testicle, showing increase in the size and number of the interstitial cells, and spermatorrhexis. Cat killed after 5 weeks.

Plate 55.

Fig. 5. Parotid glands, showing swelling of the inoculated (left) side at autopsy. Cat killed after 13 days.

776

THE ETIOLOGY, MODE OF INFECTION, AND SPECIFIC THERAPY OF WEIL'S DISEASE (SPIROCHÆTOSIS ICTEROHÆMORRHAGICA).

By RYOKICHI INADA, M.D., YUTAKA IDO, M.D., ROKURO HOKI, M.D., RENJIRO KANEKO, M.D., and HIROSHI ITO, M.D.

(*From the First Medical Clinic of the Imperial University in Kyushu, Fukuoka.*)

Plates 56 to 62.

(Received for publication, December 1, 1915.)

INTRODUCTION.

In this communication we have summarized and in some details extended the publications on this subject which have appeared in the Japanese literature.[1] In the course of our investigations of that endemic disease of portions of Japan, which agrees clinically with Weil's disease, so called, we discovered a spirochætal microorganism which is now believed to be the cause of the disease. In the experiments recorded in this paper, Drs. Inada, Ido, Hoki, and Ito chiefly conducted the experiments on animals, and Dr. Kaneko the pathological and anatomical observations.

In the western part of Japan there prevails an epidemic and endemic disease characterized by conjunctival congestion, muscular pain, fever, jaundice, hemorrhagic diathesis, and albuminuria, which is known as Weil's disease or febrile jaundice. A similar disease is also present in Shikoku and is called *Odan-eki;* that is, icteric pestilence. At the end of last year (1914) the same disease was observed in Chiba, in the eastern part of Japan, near Tokyo, where the patients numbered 178. Thus this disease appears to be prevalent in various parts of Japan, although to a small extent.

We have been interested in Weil's disease in Kyushu for many years, and at the end of November, 1914, we detected a spirochæta in the liver of a guinea pig injected with the blood of a patient suffering from Weil's disease. We came to the conclusion, in January, 1915, that this spirochæta is the pathogenic cause of Weil's disease, and we named it *Spirochæta icterohæmorrhagiæ.* At Chiba, a distance of more than 700 miles from Kyushu, we detected in the blood of five out of

[1] A full bibliography is appended.

six patients who suffered from this disease in 1914 a substance which has a spirochæticidal power over this spirochæta. Nishida, a physician in Kusunoki Hospital in Kochi, Shikoku, a former pupil of Inada, also detected by our method, a spirochæta in a patient suffering from *Odan-eki*, and his spirochæta was morphologically identical with ours.

From these facts we were able to identify the pathogenic cause of Weil's disease prevailing in three different parts of Japan, that is, in Kyushu and Chiba, and *Odan-eki* in Kochi, Shikoku, as *Spirochæta icterohæmorrhagiæ*. We can now say that it is an independent disease, caused by a specific microorganism, and we therefore propose to substitute the name of spirochætosis icterohæmorrhagica for the various names already in use. This name, although somewhat inconvenient, expresses both the symptoms and cause of the disease. Thus, some patients suffering from Weil's disease exhibit rarely only slight jaundice or do not show hemorrhage, and for these cases the name may seem inappropriate; but the occurrence of such light and atypical cases is a common experience among infectious diseases. We might shorten the name to spirochætosis icterica, as the jaundice is the most striking and constant symptom, but there is danger of confusing it with recurrent fever accompanied by jaundice; while, moreover, the name spirochætosis japonica does not differentiate it from other diseases caused by various spirochætæ.

Opinions differ as to whether Weil's disease in Europe and Japan are identical. The symptoms are similar, but there are some variations. The mortality of Weil's disease is 32 per cent in our clinic; splenic enlargement is rare (10 per cent in our clinic). Spirochætosis icterohæmorrhagica is present endemically in various localities, but rarely causes a sudden epidemic, as is the case with the European Weil's disease. On the other hand, the European Weil's disease appears either as a transient epidemic or endemically, and in the latter case the state of its outbreak and symptoms resembles the Japanese disease closely. Thus the above question can only be solved by the detection of the presence or absence of the spirochæta in the European Weil's disease.

Bilious typhoid fever resembles the Japanese Weil's disease. According to Griesinger and others, this disease is a form of recurrent fever accompanied by jaundice; but Kartulis, Diamantopulos, Goldhorn, and Rabinowitsch believe that it has no connection with recurrent fever. Goldhorn states that Weil's disease is a light form of bilious typhoid, and Weil himself called attention to the similarity of the symptoms in the two conditions. Fiedler reports that pathological anatomical changes in Weil's disease are the same as those in bilious

typhoid. The mortality of bilious typhoid, according to Griesinger, is 60 to 70 per cent; compared with that of our cases of so called Weil's disease this is high. A few years ago Nishi identified Weil's disease with yellow fever, but he was opposed by Ohno. We have also emphasized the differences from the epidemiological point of view between yellow fever and Weil's disease, although the symptoms are very much alike. The poison in Weil's disease is sometimes capable of filtration through a Berkefeld filter, as is that of yellow fever, and the microbic causes of both diseases are present in the blood only in the early stage of the illness. Both diseases are prevalent in the lowlands. Yellow fever, however, is communicated to man by the *Stegomyia* mosquito, while there is at present no proof that Weil's disease is communicated by the bite of an insect.

Typical Japanese Weil's disease can be easily differentiated from catarrhal jaundice. On the other hand, very light cases or cases in which the conjunctival congestion has already passed or which have passed beyond the initial stages and show no hemorrhages may be difficult to diagnose. Clinically one never sees in catarrhal jaundice such marked congestion of the conjunctiva as is present in the Japanese Weil's disease, and the general condition of the patients shows great differences in the two diseases. In Weil's disease the patient is weak and cannot stand up even on the 1st or 2nd day of the illness, while in catarrhal jaundice one does not meet with such symptoms. The state of the circulatory system and other symptoms make further differentiation possible. However, the two diseases may not be differentiated through the presence or absence of bactericidal substances in the blood of the patients.

Spirochæta icterohæmorrhagiæ.[2]

In February, 1915, we announced the discovery of the spirochæta of Weil's disease. It may be interesting, therefore, to review briefly the reasons for the failure to detect the spirochæta at an earlier period: (1) All investigators searched for bacteria. (2) The spirochæta resides in the blood only during the early stages of the disease and is present there in small numbers. (3) At the period at which fatal cases come to autopsy the liver is either devoid of spirochætæ or they are so few or modified in form as to be difficult of discovery or recognition.

In our early studies we searched for bacteria in the blood, urine, and feces. Having failed in this quest, we inoculated the monkey, rabbit, rat, and guinea pig with the blood of patients, and in July, 1913, we observed that the guinea pig developed albuminuria, conjunctival congestion, jaundice, and hemorrhages when the blood was

[2] This part of the investigation was conducted by Drs. Inada and Ido.

injected during the first 7 days of the illness. We ascertained later that the number of spirochætæ in the blood is so small as to make their detection under the microscope almost impossible. In the guinea pig they multiply freely and are found in large numbers in the liver. We first detected the spirochætæ in this manner in November, 1914. Inasmuch as the injection of the blood of healthy persons or even of patients suffering from other diseases including catarrhal jaundice does not cause pathologic changes in the guinea pig or lead to the occurrence of spirochætæ in the liver, we concluded that the spirochætæ were derived from the blood of the cases of Weil's disease. Our deductions were confirmed when we discovered the same spirochætæ microscopically in six specimens of patients' blood, in the intestinal wall of one case, and in the adrenal glands of another among eleven autopsied cases. The autopsies were performed on cases which had succumbed on the 8th to the 14th day of illness, the most fatal period of the disease. At this period the spirochætæ have already diminished greatly in numbers. At this time we also detected a bactericidal substance for the spirochætæ in the blood of patients, and we assumed that the disappearance of the organisms from the body was connected with the formation of this substance. Probably in this way is explained either the entire absence or the occurrence merely of atypical forms in small numbers in the organs. On the other hand, we detected later in the liver of two patients who succumbed on the 6th day of illness spirochætæ as numerous as in the liver of the guinea pig. These facts led us to assert that the spirochæta is the pathogenic cause of Weil's disease.

EXPERIMENTAL.[3]

Hecker and Otto injected defibrinated blood of soldiers suffering from Weil's disease in Europe into the skin, peritoneum, and veins of rats, guinea pigs, rabbits, and monkeys, with negative effects. Fiedler and Schittenhelm report similar results. We have injected blood of seventeen cases of spirochætosis icterohæmorrhagica into the peritoneum of the guinea pig. Thirteen positive results with typical symptoms,—conjunctival congestion, jaundice, hemorrhage, and albu-

[3] These experiments were carried out by Drs. Inada, Ido, and Hoki.

minuria,—were obtained. The blood of all the patients in the 4th to the 5th day of the illness gave positive results, while that taken on the 7th day in one instance gave positive and in another negative results. The blood in one case, on the 9th day of illness, also gave a positive result; but in no instance from the 12th day on was a result obtained, although blood from three such cases was tested. Thus, in order to obtain positive results one must inject the blood within the first 7 days of the illness. The symptoms following the injection are similar to those after inoculation from one guinea pig to another, except that the incubation period is longer. The incubation period may be reduced by making the inoculation from a patient suffering from severe illness in its early stage.

The time required after the inoculation for the appearance of jaundice is usually 7 to 8 days. The shortest time was 6 days, and the longest 13 days. The infection can be transferred from an infected guinea pig to others through many generations, and the oldest of our six strains of spirochæta has now reached the fifty-first generation (July 15, 1915). The inoculation succeeds by intraperitoneal, subcutaneous, or oral injection of 2 cc. of the heart's blood or of an emulsion of the liver. The former is the most certain. The method of inoculation also affects the incubation period. The animals succumb in from 5 to 8 days after intraperitoneal, and in from 9 to 10 days after inoculation through the uninjured skin or the alimentary canal; after inoculation into the injured skin death follows in 7 to 8 days. The spirochæta occurred in the animals experimented upon practically constantly. Of the large number of experiments made, in only two were they discovered in the liver, although jaundice and hemorrhage were present.

The symptoms produced in the guinea pig by inoculation are similar to those occurring in man. The animals show loss of appetite, conjunctival congestion, anemia, jaundice, hemorrhagic diathesis, and albuminuria. The fever comes on suddenly, reaching nearly 40°C. on the 4th or 5th day after intraperitoneal inoculation, and on the 5th or 6th day after the inoculation through the injured skin. The spirochæta appears in the blood about this time and the animals generally succumb within 24 hours after the appearance of the jaundice. Just before death the temperature falls rapidly, as in collapse.

Rabbits fail to become infected even when large quantities of the blood capable of producing the disease in guinea pigs are injected. In our experiments,

death never followed the intraperitoneal or intravenous inoculation of 20 to 40 cc. of blood or liver emulsion of the guinea pig containing the spirochæta, the fatal dose for the guinea pig being 4 to 5 cc. However, eleven of the thirteen rabbits showed a rise of temperature on the 4th or 5th day. Conjunctival congestion was present in six, and a slight jaundice in four animals. All the animals became excited. One which showed marked jaundice showed no hemorrhages or spirochætæ in the liver.

Fourteen mice were inoculated with the liver emulsion or blood of the infected guinea pig, of which four showed jaundice and slight hemorrhage and succumbed. One was examined, and no spirochætæ were found in the liver.

Two white rats were similarly inoculated. One developed jaundice and slight hemorrhage and died on the 8th day; the other remained healthy.

From the above experiments the rabbit appears to be the most insusceptible, the mouse and white rat coming next. The guinea pig is the most susceptible; it appears to be even more susceptible than man.

Pathological Changes in the Guinea Pig.[4]

The typical pathological changes consist of marked general jaundice, hemorrhages into the various parts of the body, and parenchymatous changes of the organs. The usual sites of hemorrhage are the lungs, intestinal walls, retroperitoneal tissues, and the fatty tissue of the inguinal region. When the changes are atypical there are either very slight or no hemorrhages, although the jaundice is marked, or there is no jaundice although hemorrhage is present. The latter condition is rare and generally when met with the spirochætæ are few in number or absent. After the injection of salvarsan this condition is found.

The liver shows cloudy swelling of the parenchyma, while the color varies according to the degree of the jaundice and the quantity of blood present. Microscopically the precipitation of the bile is not marked in spite of the presence of jaundice, and there is no congestion of the bile in the biliary duct.

The kidney shows an acute parenchymatous nephritis. The spleen is more or less enlarged, congested, and hemorrhagic. There is always some precipitation of the iron-containing pigment and the phagocytic cells contain red blood corpuscles. The lymphatic glands do not undergo enlargement, except those near the site of inoculation.

[4] This study was made by Drs. Inada and Kaneko.

The lungs present small and large hemorrhagic spots, like the wing of a mottled butterfly. This change is one of the most important in the diagnosis of the disease.

The intestines show hemorrhages in the walls. The muscles show a certain amount of hemorrhage and parenchymatous changes of the muscular fibers.

Distribution of the Spirochætæ.

The spirochæta lives in the blood outside the cellular elements and in various organs and tissues. When present in the cells it is within phagocytic cells. It is often seen in the epithelial cells. The liver contains the largest number of spirochætæ. They can be demonstrated most clearly by the older Levaditi method of silver impregnation. They occur usually in the spaces between the cells, and when present in large numbers are arranged about the individual cells like a garland. Next to the liver the adrenal glands and the kidneys contain the largest number of spirochætæ. In the kidneys the spirochæta occurs inside the interstitial tissues and also in the walls and lumen of the uriniferous tubules.

There are very few spirochætæ in the spleen, bone marrow, and lymph glands, although they are rich in blood; and the spirochætæ are extremely rare in the splenic follicles, as well as in the lymph glands and other lymphoid tissues. Diffuse hemorrhages in the lung and intestinal walls may contain only a small number.

With a given strain of spirochæta, the animals that show the most marked pathological changes have the most spirochætæ. Among the six strains of spirochætæ, two, which were obtained from severe cases, produced severe pathological changes associated with more numerous spirochætæ than followed the inoculation of the other strains. The pathological changes produced by the above two strains have, however, become gradually reduced as successive transfers have been made.

Comparison of the pathological changes in the guinea pig with those of man brings out certain differences: (1) The hemorrhage is severer and more wide-spread in the lungs of the guinea pig than in man. (2) Spirochætæ can always be found in the guinea pig when the inoculation gives a typical result, which is not the case in man. This

is the most important difference between the human disease and the infection in guinea pigs. (3) The spirochætæ are present within the liver cells in man, and lie between the liver cells in the guinea pig. This difference may be accounted for by the fact that no immunizing substance is formed in the guinea pig. In man a bactericidal substance injurious to the spirochæta appears in the blood on the 14th or 15th day of illness. Moreover, man is more subject to intercurrent mixed infections than the guinea pig.

Characteristics of the Spirochœtæ.[5]

Spirochœta icterohœmorrhagiœ remains always outside the blood cells. It resides in the interstitial tissues of the organs and rarely in epithelial or phagocytic cells. Like that of recurrent fever it probably belongs to the blood spirochætæ. This view is supported by the acute nature of the disease and by the fact that the spirochætæ of chronic diseases like syphilis and frambesia belong to the group of tissue parasites. Although it is impossible to measure the length of the spirochæta exactly, because of its irregular wavy figure, the specimens seen in blood taken from a case of Weil's disease varied from half the diameter to the full diameter of the red corpuscles. The measurements are the same in the blood of the guinea pig. The commonest length is between 6 to 9 μ, the greatest reaching 12 to 13 μ. In the liver the short forms measure from 4 to 5 μ; but the common forms are longer and measure 8 to 9 μ. The longest individuals average 20 μ, although we have seen one measuring 25 μ.

The thickness varies according to the staining and fixing methods and the strength of the stain. The thickness is probably 0.25 μ. The ends are sharp and in most cases hooked. In some specimens one end only is hooked, and in others both ends are bent toward the same side, resembling the letter C; or the ends are turned opposite to each other forming the letter S. The undulations are not so regular as in *Treponema pallidum* and are usually composed of two or three large irregular, or four or five smaller waves.

Flagella cannot be demonstrated by Loeffler's method, and it is probable, therefore, that a membrane is absent. This point is not established.

[5] This study was made by Drs. Inada and Ido.

The methods employed in staining are as follows: Liver and blood specimens are fixed with absolute alcohol, methyl alcohol, or osmic acid (Weidenreich's method), and the staining is done with Giemsa's solution. The specimen fixed with methyl alcohol is stained for 2 hours or over with a mixture of about 2 cc. of water and three drops of Giemsa's solution. The spirochæta is usually well stained when the granules of the white blood corpuscles are deeply stained. The color of the spirochæta varies according to the degree of the staining, and is either red or red with a purplish tinge. For vital staining 50 per cent borax methylene blue is employed.

The spirochætæ stained in this manner look uniform, but under high magnification some individuals show folds and present the granular appearance seen under dark-field illumination. Moreover, some of the spirochætæ show the granular appearance distinctly when stained by Loeffler's method.

The spirochætæ in the blood usually show the typical and rarely the atypical form. But when they are very numerous, the atypical forms—ring form or two twined about one another—are more frequent.

The forms present in the liver are as variable as are the differences in length. One sees round or oblong granules, sometimes three or four in number, stained deep purple with Giemsa's solution in the body of the spirochæta. These granules appear to be collections of chromatin. In addition still larger granules sometimes project from the body of the organism forming the so called bud of the spirochæta. As in the case of the other spirochætæ, the significance of this bud is not apparent. Some of the spirochætæ resemble a platinum loop at one end or appear as rings, in which case the ends are not visible. The degenerative form is thick and straight, devoid of waves, and blunt at the ends.

The spirochætæ are not visible in the unstained condition under the microscope, even when very numerous. Under dark-field illumination they are readily found and present a characteristic appearance. The light is refracted unevenly, and portions showing strong light alternate with portions showing no light. The latter are narrower than the former, giving to the organism the appearance of a rosary. According to the length of the spirochæta the refractive

granules number 25 to 30 to 40. There are fore and aft movements besides movements of the ends to right and left. The anterior third of the spirochæta makes brisk movements to the right and left, while the posterior part makes only slow movements with the end turned either to the right or left. There is also a twisting motion about the long axis, which makes the spirochæta look like a figure 8, and there are spiral or snake-like movements, besides the formation of waves along the long axis, as if the waves of muscular contraction were transmitted to the spirochæta.

When a liver emulsion is left at room temperature, the spirochæta keeps its movements for 2 days, although they are not brisk, at the end of which period the majority are precipitated to the bottom of the culture tube.

The spirochæta immediately ceases to move when put in 50 per cent glycerine, 5 per cent sodium chloride, or 0.5 per cent acetic acid solution; it looks as if it were hardened and shows various degenerative changes, such as a bending small ring formation at one or both ends. 0.5 per cent sodium hydrate solution immediately dissolves the spirochætæ, but they continue to move for a comparatively long time in distilled water.

Filterability of the Spirochætæ.

The results of experiments conducted with five Berkefeld V, one N, and three W candles were variable. The filters had previously been tested with *Bacterium coli commune*, not with *Bacillus prodigiosus*, and were found to be impervious to the former. Some of the filtrates when injected produced infection in a guinea pig, but spirochætæ were not detected under dark-field illumination.

Of twenty-eight experiments made with liver emulsion of a particular strain, fifteen positive results were obtained. As a rule, the spirochæta appears to be more easily filterable when present in large numbers.

It is difficult to determine the mode of multiplication of the spirochæta, and various opinions might be expressed, as with the mode of the multiplication of other spirochætæ. We examined many fresh specimens under dark-field illumination daily for over 6 months. On one occasion Hoki saw a spirochæta suggesting transverse seg-

mentation, and Ito and Inada saw one suggesting longitudinal segmentation. We think, however, that multiplication probably occurs by transverse segmentation, since one sees numerous short forms at times when the multiplication of the spirochæta is going on rapidly, as is described below.

Cultivation of the Spirochætæ.[6]

It was not until April, 1915, that we succeeded in keeping the spirochæta alive from 13 to 17 days. In May we observed its multiplication and were able to transmit it from generation to generation. At that time we cultivated it through five generations, and the spirochæta lived in the culture medium for 2 months after it left the body of the animal. The method employed was that of Noguchi, by means of which various spirochætæ of recurrent fever have been cultivated. We employed guinea pig instead of rabbit kidney, and always used liquid paraffin. The most important fact, however, is that the temperature of 37°C. is not suitable for its development. When a well grown culture is kept at 37°C. the movements of the spirochæta become sluggish within 2 to 3 days, and almost all the spirochætæ undergo degeneration and finally disappear from the culture medium. Temperatures below 15°C. are also unsuitable, the best results being secured at temperatures of 22 to 25°C.

The culture does not yield any odor, and the ascitic fluid remains uncoagulated. Moreover, the fluid remains clear; and even slight cloudiness indicates contamination with some coccus or bacillus. The spirochætæ are distributed almost uniformly throughout the culture medium. The life of the culture is variable at 22–25°C. The first generation lives mostly from 3 to 6 weeks, the longest period observed being 55 days and the shortest 17 days. The life of the second and third generations is somewhat shorter than that of the first generation. The conditions which conduce to long life are at present not established. When extraneous bacteria are present in considerable numbers in the culture the spirochætæ die, but they can survive if the number of foreign bacteria is small, although they will not grow when transplanted. Thus protection from contamina-

[6] This work was done by Drs. Inada and Ido.

tion is an important factor in the cultivation. The multiplication of the spirochætæ in the culture starts at different periods, sometimes after 2 to 3 days and at other times only after 1 week or, rarely, after 2 weeks. In the transfer of the culture from one tube to another the addition of a small quantity of blood keeps up the development, while the best time for the transfer is when multiplication is going on rapidly, as is indicated by an examination of the culture every 2 or 3 days.

In character the cultivated spirochæta does not differ from that obtained directly from the animal body. When multiplication is at its height the spirochæta is very short and has brisk movements. In the young culture over half the spirochætæ may be composed of the short forms which, however, gradually become longer. Sometimes two spirochætæ are connected, and again one sees 8 to 9 to 15 spirochætæ collected about some granular substance in a manner resembling a rosette, with very brisk movements. Rarely very long spirochætæ (two or three times the normal length) composed of a single organism may occur. After the height of development the movements gradually become sluggish, the spirochæta takes on a hardened and bent appearance, assumes various degenerative forms, and finally dies. The second and third generations of the pure culture are capable of producing on inoculation infection in the guinea pig; whether later generations do so has yet to be determined. In the absence of a simpler method, we have successfully employed Noguchi's method of cultivation.

Mode of Infection.[7]

Weil believed that in the European Weil's disease infection occurred through the alimentary canal. Fiedler held the same view, but Hecker and Otto believed that infection might be communicated by the bite of a mosquito. As regards Weil's disease in Japan, Inada thinks that the infection occurs probably through the alimentary canal. It may, however, sometimes enter through the skin, as at times the disease begins with local swelling of the lymph glands. The throat is probably not the place of invasion, although it may show congestion. Oguro in Saga, on the other hand, has never observed

[7] This study was made by Drs. Ido and Hoki.

any circumstance which suggests the cutaneous mode of infection, but regards the alimentary tract as the portal of infection. Thus the mode of infection cannot be considered as established from clinical observation. On the other hand, since the discovery of the pathogenic cause of the disease, we have been able to determine it by experiments on animals.

The abdominal wall of the guinea pig is shaved without injuring the skin, washed with soap and then with alcohol, and dried. 1 cc. of the liver emulsion containing the spirochæta is dropped on its surface with or without abrading it. Thirty guinea pigs were so treated after incision of the abdominal skin to the extent of causing slight bleeding, and thirteen animals without injuring the skin. 10 of the latter 13 animals (77 per cent) and 26 of the first 30 (86 per cent) contracted the disease. Thus one can see that the spirochæta is able to penetrate through a macroscopically healthy skin and cause the disease in the guinea pig. The invasion occurs more easily and certainly where an obvious lesion exists. The incubation period, moreover, is 9 to 10 days in the former, and 7 to 8 days in the latter instances. Crushed liver acts as well as the emulsion for the purpose. The time required by the spirochæta to penetrate through the skin is short, and even after the skin is washed with alcohol or sublimate solution, the animals acquire the disease 5 minutes after the application of the emulsion to the skin. Whether penetration takes place in less than 5 minutes has not been determined. The spirochæta can also be made to invade the body through the alimentary canal. Feeding 2 gm. of liver emulsion, or giving an enema of 2 gm. of liver emulsion containing the spirochæta, produces the disease in the guinea pig. Thus, the spirochæta is able to invade animals through the mucous membrane of the alimentary canal.

Among fifty-five cases which were admitted to our clinic, only a few indicated cutaneous origin. The following facts, however, suggest this mode of infection: (1) When the disease occurs in coal mines the patients are numerous among the miners who work at a certain part of the mines. Numerous cases do not arise in the same barracks, even when the disease originates there. (2) The clerks working outside the mines do not contract the disease. (3) There are many cases in wet mines and few in dry mines. (4) It was noted that many cases occurred among the miners who worked in a particular part of the mine, but no case occurred when the accumulated water, as suggested by Inada, was pumped out. As mentioned above, the spirochæta is able to penetrate a healthy skin. Thus anyone who is working in an infected locality can easily contract the dis-

ease, even when the skin is uninjured, and the infection will take place still more easily if the skin is injured. Coal miners, who are liable to abrasion of the skin, and also to a skin lesion caused by working with the feet in water, can easily contract the disease.

We thought that infection through the skin was probably on account of the presence of enlarged lymph glands, but, later, after observing numerous cases, we considered the enlargement of the glands as a result of the general infection and not the result of the direct invasion of the spirochæta into the glands. However, from the animal experiments described above, we believe that the enlargement is partly due to the result of local infection of the glands in the affected part and partly to the general infection. The enlargement of the glands is not a symptom which is present in every case; it was present in about 60 per cent of fifty-five cases admitted to our clinic. The enlarged glands usually reach the size of a horse-bean or less. We have only once seen the gland as large as the end of the thumb. Besides skin infection, infection through the alimentary canal possibly occurs also. Cases arise in which two or three persons in a family are affected at the same time or at an interval of 1 or 2 days. However, we do not yet know how the infection in these instances occurs.

Since the origin of the infection through the skin is established, it is necessary to consider the question of whether the mosquito or flea may play a part in causing it. Among our clinical cases indication of direct infection from man to man is rare; while the relation of the seasons and the fact that the disease is common in Hakata and Chiyomachi and rare in Fukuoka on the other side of the river, make us think that the mosquito and flea do not play a part in the propagation of the disease.

The spirochæta of the Japanese Weil's disease is not present in such large numbers in the blood as is the spirochæta of recurrent fever; the number diminishes rapidly and they disappear comparatively soon from the blood. Moreover, the virulence in man is less than in the guinea pig, and wide-spread epidemics, as in recurrent fever, do not occur.

Mode of Excretion of the Spirochæta.[8]

In order to detect the mode of excretion of the spirochæta, it is necessary first to examine the excretions with dark-field illumination and to inoculate guinea pigs with them. This double procedure is important, for even when the spirochæta cannot be recognized with the ultramicroscope, the animal experiments may prove positive. Ida, of the Second Medical Clinic of Kyushu University, inoculated urine from a case of Weil's disease into a guinea pig and applied some of the urine to the skin of the animal, after injuring the surface. He obtained a positive result. Later we visited several coal mines and examined the excretions under the dark-field microscope. We conducted experiments with animals at various stages of the disease with twenty-four cases.

In twelve cases we examined the urine with the dark-field microscope within the first 10 days of illness, and recognized a small number of spirochætæ, in one case on the 6th and in another on the 10th day. The others gave negative results.

It is noteworthy that numerous spirochætæ are found in the urine at a time when the immune body appears in the blood; that is, about the 13th to the 15th day of illness. In seven cases from the 10th to the 30th day of illness, we found numerous spirochætæ in the urinary sediments of the five patients in which a negative result was obtained during the first 10 days. In four of these five patients the microscopic field contained countless spirochætæ. The spirochætæ were chiefly present in the cylindroids and nubeculæ, and in small numbers in the cylinders. We studied the spirochætæ in two of these five cases and found that they begin to degenerate and finally disappear from the urine altogether before the 40th day of illness. Later we studied the urine from ten cases after the 30th day of illness and detected a small number of degenerative spirochætæ in two on the 32nd and 38th days, respectively. The remaining eight cases gave negative results.

Hence the urine contains the spirochæta, beginning at an early stage of the disease, but in such small numbers as to make detection by the dark-field microscope difficult. However, from the 13th to

[8] This work was done by Drs. Ido, Hoki, and Ito.

the 15th day of illness, at the time that the immune substance appears in the blood, they become numerous and are easily discovered. After the 24th to the 25th day the spirochætæ begin to diminish in number and to assume a degenerative form, while, in our experience, they disappear completely from the urine before the 40th day of illness. However, these points require further investigation before a definite conclusion can be reached.

The results of the inoculation of urine into guinea pigs are given in Table I.

TABLE I.

Material used for inoculation.	No. of inoculations.	Positive result.
Urine from 5 patients before the 10th day of illness	9	3
Urine from 4 patients between the 11th and 20th days of illness	15	5
Urine from 9 patients after the 31st day of illness	12	0

The inoculation of the urine taken during the first 10 days of illness gave results in one-third of the cases, even though it was difficult to detect the spirochæta in the urine; and, conversely, although the urine contained many spirochætæ after the 13th to the 15th day of illness, the inoculations gave only the same percentage of positive results. Possibly the immune substance, which begins to appear in the urine about the 24th or 25th day, may bear upon that phenomenon.

Feces from eight patients were inoculated into guinea pigs on ten different occasions with one positive result, from a patient on the 7th day of illness.

Sputum from two patients was injected into guinea pigs on three occasions. A positive result was obtained once in a case in which the sputum was bloody. Two inoculations with vomitus gave no result.

Manner of Excretion in the Experimental Disease.

On examination of the urine, feces, and the contents of the gallbladder in twenty-five infected guinea pigs, a small number of spirochætæ was detected in the urine by the dark-field apparatus in eight

of the thirty-two examinations. The examinations of the contents of the gall-bladder were all negative; while those of the feces and intestinal contents gave microscopically a positive result in one of twenty examinations.

The inoculation test with the materials mentioned gave a wholly different result: the urine produced infection in 7 of 9 tests, the feces and the intestinal contents in 7 of 11 tests, and the contents of the gall-bladder in 2 of 3 tests. Thus, the spirochæta is excreted in the urine, feces, and bile in the experimental disease of the guinea pig up to the time of death, although the number excreted in the urine is small.

The following experiments were made in order to determine whether the excretion of numerous spirochætæ in the patient's urine in the convalescent stage of the disease is related to the appearance of the immune substance in the blood.

Experiment 1.—The distribution of the spirochætæ in the various organs was studied after immune goat serum was injected into the guinea pig at the time of the appearance of jaundice.

Experiment 2.—The urine from the guinea pigs which contracted the disease from cutaneous inoculation and which had recovered without developing jaundice under treatment with immune goat serum, was examined daily at the time when the spirochætæ were present in the blood. The animals were killed about 20 days after the injection of the immune serum, and the distribution of the spirochætæ in the body was studied.

The first experiment was limited to two animals, and no spirochætæ could be discovered in the liver under the dark-field microscope, although the kidney showed a small number.

The second experiment was performed with four animals. In two that passed the 10th and 13th days respectively after the injection of the immune serum, the spirochæta was recognized in the urine. All four animals were killed on the 20th day after the serum treatment, and the blood, liver, kidneys, and urine were examined under dark-field illumination. The blood and liver showed no spirochætæ, while the kidneys and urine in the bladder showed typical spirochætæ with active movements.

In the experimentally infected guinea pigs the number of the spirochætæ excreted in the urine is usually small, and this is probably due to the circumstance that the animals die before the immune substance is formed in the body. This is indicated by the fact that the spirochætæ appear in the urine in the animals which are treated with the immune serum. The serum treatment probably confers an immunity upon the animals.

Whether the formation of the immune substances which appear to limit the development of the spirochætæ in the body and cause their collection in the kidneys and hence excretion into the urine is responsible for the appearance of the large numbers in the urine in human cases at the time of convalescence, is not clear. Perhaps the spirochætæ are merely able to multiply in the kidney for a time, after which they are excreted. The autopsies indicate that these phenomena occur. The immune substances, moreover, are present in the urine as well as in the blood for a certain period after the 24th or 25th day of illness. When the immune bodies begin to appear in the urine the spirochætæ gradually degenerate and disappear.

Immunity Phenomena.[9]

In the spirochætal disease under consideration a substance which has bactericidal and bacteriolytic action over the spirochætæ appears in the blood. The discovery of this substance was of great importance in the detection of the spirochætæ. The existence of the immune body can be demonstrated by Pfeiffer's method, as follows:

1 to 2 cc. of liver emulsion, containing spirochætæ, are injected into the peritoneal cavity of guinea pigs. In one series of animals the patient's serum is also injected, while in the control animal salt solution or the serum of a healthy person is employed for the injection. After ½ to 2 hours peritoneal fluid is withdrawn and examined microscopically. The fluid from the control animals shows numerous spirochætæ with active movements, although in somewhat diminished numbers after 2 hours, while after half an hour the fluid from the animal treated with the patient's serum will be free of spirochætæ.

Moreover, the subsequent condition of the animals confirms the presence of the immune body. Those in which the spirochætæ disappear from the peritoneal fluid after half an hour usually do not develop the infection, while those which still show after 2 hours a small number of spirochætæ in the peritoneal fluid develop jaundice and hemorrhage and finally die. These phenomena occur both with active and inactive serum.

The immune body exerts its action when injected along with the spirochætæ and also when the spirochætæ are already in the tissues. When the serum of a convalescent patient is injected into a guinea pig which has already developed jaundice and hemorrhage, the spiro-

[9] This work was carried out by Drs. Hoki and Ito.

chætæ disappear from the blood after half an hour, and after $3\frac{1}{2}$ hours only a small number of the degenerative type of the organism can be detected in sections of the liver by Levaditi's method of staining. At the end of 8 hours no spirochætæ are seen, but in these instances we were able to find a few spirochætæ in the other organs, especially the kidneys and suprarenals, by using the silver impregnation method. Identical results were obtained with each of the six strains of spirochætæ that we now possess.

In order to determine the time of appearance of the immune substance, sixteen cases of Weil's disease were examined within the first 30 days of illness. The majority of the sera examined had been preserved for 2 years. Pfeiffer's reaction proved positive in one case on the 14th day, in two cases on the 15th day, in one case on the 22nd day, and in three cases between the 24th and 28th days of illness.

These results show clearly that the immune substance is fully formed about the 14th to the 15th day of the illness. On the other hand, examination of eight cases from the 4th to the 11th day all proved negative, while one case was negative on the 9th day and positive on the 20th day, and another was negative on the 7th day and positive on the 15th day of illness.

The immunity endures for a long period of time. Of thirteen cases examined later than a month since the illness, immune bodies were detected at the expiration of over $5\frac{1}{2}$ years (two cases), 3 years and 2 months (one case), 2 years and 4 months (two cases), and 1 year and 10 months (one case). This immune substance is specific in Weil's disease and is not present in the serum of healthy persons who have not had Weil's disease or in persons with jaundice of other nature.

Distribution of the Spirochætæ in the Human Body.[10]

Postmortem examinations were performed on twelve cases which died between the 9th and 16th days of illness. Tissues from eight cases were fixed in formalin solution, and tissues from one case which died on the 8th day of illness had been preserved in Orth's solution for 8 years. The examination showed that while the kidneys contain the spirochætæ in the largest number, yet in every case spirochætæ were detected.

[10] This work was done by Dr. Kaneko.

Spirochætæ in the kidneys were mostly within the uriniferous tubules, and the majority were contained in the substance of urinary cylinders (casts). However, a small number occurred within the detritus in the uriniferous tubules or was attached to the epithelial cells. In tissues from one case which succumbed on the 10th day localized accumulations of the spirochætæ in the act of invading the tubules were seen in the interstitial tissues. The spirochætæ in the urinary cylinders were most numerous in a case succumbing on the 9th day, and within the lumen of the uriniferous tubules in a case succumbing on the 14th day.

The liver showed spirochætæ in smaller numbers, of which many were degenerated. No spirochætæ were found in the specimen of liver preserved in Orth's solution. Within the liver the spirochætæ were chiefly present within the hepatic cells; they occurred frequently also at a comparatively early stage of the disease in the interstitial tissues and often also in the stellate cells of Kupffer. In cases dying about the 9th day the spirochætæ were typical in form and occurred within the veins and on the outer surface of the cells, while in cases from the 14th to the 16th day of illness they were present only within the hepatic cells. The distribution of the spirochætæ in the suprarenal gland is similar to that in the liver.

The lymph glands and spleen contained a small number of spirochætæ, mostly in a degenerated condition. They were chiefly inside of phagocytic cells, while comparatively well preserved organisms were seen in masses of coagulated blood. This condition of the lymph glands is to be distinguished from what occurs in locally infected glands; in the latter in six cases examined typical spirochætæ existed, although in small number, while in the autopsied cases typical organisms rarely were met with.

As regards the other organs, comparatively well preserved organisms were found in the cardiac muscle, voluntary muscle, testicle, and arterial walls of the autopsied cases. The cardiac muscle especially exhibited well preserved, typical spirochætæ within the cell bodies and in the interstitial tissues. The spirochætæ were detected at times in the lung, pancreas, intestines, gall-bladder, genitals, nervous system, and skin, but they were mostly degenerated.

Thus the distribution of the spirochætæ in the human body differs from that in the guinea pig in that the number present is smaller, the degenerative forms are more abundant, they are more within cells, the kidney contains more, and the liver and adrenal glands contain fewer; the cardiac muscles, voluntary muscles, and arterial wall contain typical organisms.

The above differences can probably be explained by the action of the immune body, which appears in the course of the disease. This immune body destroys and dissolves the spirochætæ, and consequently one finds a smaller number of them in man. The greater occurrence of intracellular organisms is probably due to the fact that the spirochætæ invade cells in order to escape from the action of the immune body; while the presence of the spirochætæ in the epithelial cells of the glandular diseases perhaps indicates its way of escape from the body. The fact that the number of spirochætæ found in the autopsied cases is small may be explained partly by imperfect preservation of tissues and mixed infection; and we believe, therefore, greater numbers will be detected by means of a perfected method of study in the early stages of cases of pure infection. This view has recently been supported by finding the liver in two cases, which died on the 6th day, as heavily infected with spirochætæ as in the guinea pig.

Prophylaxis.

The mode of infection of the disease and the excretion of the spirochætæ indicate the means and the necessity of prophylaxis. As already mentioned, the disease occurs frequently among coal miners who work in that part of a mine which is inundated with water got rid of by pumping. It is, therefore, necessary to remove the water and then to disinfect the ground, when it is found that the disease occurs among the workmen who work at certain definite places in the mines. Lime is effective for the disinfection of the ground. It is also necessary to avoid infection through the healthy skin and the alimentary canal. Thus, when the infected locality is large so that it cannot be disinfected thoroughly, recourse must be had to the method of active immunization. Our study of this question is still under way, so that results can only partially be mentioned here.

Guinea pigs were immunized with repeated injections of liver emulsion of the infected animal and later with a pure culture of the spirochætæ which had been killed by carbolic acid. The animals thus immunized did not develop the disease on the injection of the spirochætæ, which, it was known, would produce the disease in healthy animals. Hence this method seems promising for the prevention of the disease in man. Our conclusion is that the flea and mosquito have no share in the infection.

Moreover, it is necessary to disinfect the urine for at least 40 days from the beginning of the illness, in view of the fact that the spirochætæ are incessantly excreted in the urine during the course of the disease, and even as late as the 40th day. Although the spirochætæ appear to be in small numbers only in stools, they should be disinfected, as should also bloody sputum.

The spirochætæ seem to live in water and mud and to invade the human body from there; hence it is important to study the conditions necessary for their existence and especially the question of temperature, and to discover the existence of the spirochæta carrier.

Experimental Therapeutics.

Treatment with Salvarsan.—Guinea pigs were used for this purpose. The method and time of the inoculation with the spirochætæ were different in various cases. We could not discover the spirochæta in the liver under dark-field illumination in fourteen out of nineteen guinea pigs which underwent treatment with salvarsan (0.1 gm. of salvarsan per kilo of body weight), but found jaundice and hemorrhage in six instances after inoculation of an emulsion made from livers in which spirochætæ were not found.

Of twenty-one guinea pigs which were treated with salvarsan (0.05 gm. of salvarsan per kilo of body weight), dark-field illumination showed spirochætæ in nine instances, and inoculation from the nine cases gave positive results in seven instances.

Examination of the blood for spirochætæ before and after the salvarsan injection was done in seven animals. Three cases proved negative after the injection, although they showed spirochætæ in the blood before the injection. In one animal spirochætæ were absent before the injection and were present for a day after the injection.

The other three animals proved negative both before and after the injection. From these results it may be concluded that the spirochætæ are removed from the blood by salvarsan.

The action of salvarsan against the spirochætæ in the liver and other organs is still under investigation and will be reported later. The animals often died from 4 to 8 days after salvarsan injection. Professor Hata informs us that this is due to the fact that the resistance of the guinea pig against arsenical compounds is low. This weak resistance to arsenic is unfortunate, since the guinea pig is the only animal possessing strong susceptibilities for the spirochætæ.

Treatment with Immune Serum.—At first we used serum taken from a convalescent patient, and later serum from an immunized goat. The treatment with both sera gave better results than did the salvarsan treatment.

1 to 2 cc. of the serum from the convalescent patient were injected, before the appearance of jaundice, into six guinea pigs. Five of them developed no typical symptoms and no spirochætæ were found in the liver.

One of the animals developed hemorrhage and jaundice, but upon examination of the liver no spirochætæ were found. Six animals which received the serum injection, after the appearance of jaundice, showed postmortem typical changes, but spirochætæ were present in the liver in two instances only.

Immune goat serum was injected into twelve animals before the appearance of jaundice and at the time when the fever developed and spirochætæ were present in the blood. The spirochætæ disappeared from the blood half an hour after the serum injection and all the animals remained alive, except one which died on the 3rd day. This animal showed typical postmortem changes, but spirochætæ were not found in the liver, and the inoculation test with the liver emulsion also proved negative. Two of the remaining eleven animals developed slight jaundice, but recovered.

The injection of immune goat serum is ineffective after jaundice has appeared, and the guinea pig dies after the treatment. We did not find spirochætæ in the liver, and the inoculation test was negative, as was the microscopical examination of the organ. These results indicate a great difference between salvarsan and immune serum in treating the experimental disease.

The temperature of the animals which received the immune serum before the appearance of jaundice does not fall as in collapse, but falls at first and then rises again slightly. This phenomenon resembles that of the fever following the crisis in Weil's disease.

The strength of our immune goat serum is the same as that of the patient in the convalescent stage of the disease. Whether the strength can be increased has not yet been determined.

The serum treatment of the experimentally infected animals has, so far, given satisfactory results and affords a good basis for the clinical use of the serum treatment on man. The serum treatment is not suitable for cases which suffer relapses, but may be considered hopeful for cases which have already developed an immune body in the course of the disease, with lasting immunity.

We have also immunized horses for the purpose of obtaining an immune serum and we have had at least a partial success with horse serum. In a case in which it was administered subcutaneously the spirochætæ disappeared from the blood within 24 hours.

BIBLIOGRAPHY.

Inada and Ido, A Report on the Discovery of the Causal Organism (a New Species of Spirochæta) of Weil's Disease, *Tokyo Ijishinshi*, 1915, No. 1908.

Inada and Ido, A Report on the Cause of Weil's Disease, *Tokyo Ijishinshi*, 1915, No. 1920–1921.

Inada and Ido, Weil's Disease in Chiba, *Tokyo Ijishinshi*, 1915, No. 1920.

Nishida, *Odan-eki* and Its Cause, a Spirochæta, *Tokyo Ijishinshi*, 1915, No. 1920.

Inada and Kaneko, A Report on the Pathology and Anatomy of Animal Experiments with Spirochætosis Icterohæmorrhagica, *Chugai Ijishinpo*, 1915, No. 849; *Nippon Naika Gakkai Zasshi*, iii, No. 3.

Inada, Hoki, and Ido, Animal Experiments on Spirochætosis Icterohæmorrhagica, *Chugai Ijishinpo*, 1915, No. 852; *Nippon Naika Gakkai Zasshi*, iii, No. 3.

Ido, Hoki, and Ito, The Mode of Excretion of *Spirochæta icterohæmorrhagiæ*, *Iji Shinbun*, 1915, No. 931.

Ido and Hoki, The Mode of Invasion of *Spirochæta icterohæmorrhagiæ*, *Nippon Naika Gakkai Zasshi*, iii, No. 3; *Iji Shinbun*, 1915, No. 932.

Inada and Ido, The Pure Cultivation of *Spirochæta icterohæmorrhagiæ*, *Saikingaku Zasshi*, 1915, No. 239.

Hoki and Ito, Immunity in Spirochætosis Icterohæmorrhagica, *Igaku Chuo Zasshi*, 1915, No. 113.

Kaneko, The Distribution of *Spirochæta icterohæmorrhagiæ* in the Human Body, *Tokyo Ijishinshi*, 1915, No. 1932–1936.

Kaneko and Okuda, The Pathological and Anatomical State of the Lymph Glands in the Early Stage, and the Distribution of the Spirochæta in Them, *Tokyo Ijishinshi*, 1915, No. 1940–1945.

Inada, Ido, Kaneko, Hoki, and Ito, Spirochætosis Icterohæmorrhagica. The Etiology, Morbid Anatomy, Pathology, Symptoms, Diagnosis, Prophylaxis, and Treatment, *Nisshin Igaku*, 1915, v, No. 1.

EXPLANATION OF PLATES.

Plate 56.

Figs. 1 to 5. Microphotographs of *Spirochæta icterohæmorrhagiæ* in human blood. Giemsa stain. × 750.

Fig. 1. H. K. 6th day of illness.

Fig. 2. I. H. 4th day.

Fig. 3. I. Y. 11th day.

Fig. 4. I. Y. 11th day.

Fig. 5. Y. T. 8th day.

Figs. 6 to 9. Spirochætæ in the blood of a guinea pig.

Fig. 6. Giemsa stain. × 750.

Figs. 7 and 8. Silver impregnation. × 1,000.

Plate 57.

Fig. 9. Two spirochætæ intertwined.

Fig. 10. Cover-glass specimen of the liver of a guinea pig. Giemsa stain. × 2,500.

Fig. 11. Spirochætæ in the liver of a guinea pig which developed the disease after an injection of *Spirochæta icterohæmorrhagiæ*.

Fig. 12. Spirochætæ intertwined.

Fig. 13. A degenerative type.

Fig. 14. Spirochætæ ending in a cluster.

Plate 58.

Fig. 15. A spirochæta in the skin of a guinea pig 10 hours after infection. The spirochæta is in the corium.

Figs. 16, 17, and 18. Spirochætæ excreted in the urine of patients.

Fig. 16. Numerous normal spirochætæ in a cylindroid from a patient (S.) on the 16th day of the disease. Dark-field illumination.

Fig. 17. Numerous degenerative types in a cylindroid from a patient (I.) on the 23rd day of the disease.

Fig. 18. Microphotograph of spirochætæ in the urinary sediment from the same patient as Fig. 16.

PLATE 59.

FIGS. 19, 20, 21, 22, and 23. Spirochætæ in the organs in spirochætosis icterohæmorrhagica.

FIG. 19. Degenerative type of spirochætæ in the liver (intracellular) of a patient autopsied on the 13th day of the disease.

FIG. 20. Spirochætæ in the kidney of a patient autopsied on the 10th day of the disease.

FIG. 21. Spirochætæ in the cylinder of the kidney of a patient autopsied on the 8th day.

FIG. 22. Spirochætæ in the liver of a patient autopsied on the 6th day. × 350.

PLATE 60.

FIG. 23. Drawing of Fig. 22.

FIG. 24. Microphotograph of spirochætæ in the uriniferous tubules of a guinea pig, which recovered after treatment with immune serum. Killed after 21 days.

PLATE 61.

FIG. 25. Forms of *Spirochæta icterohæmorrhagiæ.*

1 to 65. Giemsa stain. 66 to 87. Silver-impregnated spirochætæ from tissues. 1 to 25 and 66 to 68. Various forms of the spirochæta. 26. A spirochæta 25 μ in length. 27 to 32, 73 to 74, and 77 to 87. Various forms of the degenerative type. 33 to 41, and 43. A mass of chromatin (?) and the so called bud of the spirochæta. 42, 69, and 70. A spirochæta ending in a cluster. 44 to 47, 75 and 76. The so called bud of a short spirochæta. 48 to 51. A spirochæta which resembles a platinum loop at one end. 52 to 59, 71 and 72. A spirochæta forming a ring. 60 to 63. A spirochæta with a small ring. 64 and 65. Forms which are considered to be in the stage of multiplication.

PLATE 62.

FIG. 26. Forms and movements of *Spirochæta icterohæmorrhagiæ.* The pure culture is observed under dark-field illumination.

1. Forward motion. 2 to 4. Rotating motion. 5. Rotating motion of a less active spirochæta. 6. A snake-like movement. 7 and 8. Young, short forms. 9. Grouping in the form of a rosette. 10 to 12. A group of spirochætæ. 13. Pair-form. 14 and 15. A mass of chromatin (?). 16. A spirochæta ending in a cluster. 17 and 18. Long forms (two and three times the length of the usual spirochætæ). 19. A straight form. 20. A thick form. 21 to 29. Various degenerative forms (crooked form). 30 to 34. Other degenerative forms. 35. A transition form between the above and the normal. 36. Spiral movement.

A METHOD FOR THE RAPID PREPARATION OF ANTI-MENINGITIS SERUM.

By HAROLD L. AMOSS, M.D., and MARTHA WOLLSTEIN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 12, 1916.)

The European war has brought about a greatly increased demand for antimeningococcic serum. Epidemic meningitis is known to be one of the attendants of armies in barracks and in the field, and the present war has proven no exception to this rule. Information is at hand indicating that at least the principal, if not all the belligerent countries have suffered from epidemics of meningitis to a greater or less extent. Now that we have knowledge of the manner in which epidemic meningitis is conveyed, namely, by means of meningococcus carriers who harbor that microorganism in the nasopharynx, the appearance of meningitis in widely separated countries and places is not at all remarkable. Meningococcic meningitis has prevailed either in epidemic form or sporadically in many European countries during the past 10 or more years. Hence the bringing together of recruits has had the effect of introducing carriers of the infection into the midst of the European armies. Not until the European war is over and the medical data of the war have been collected and analyzed shall we know whether the epidemics have been of equal severity throughout or whether some have been of greater severity than others. But the interim observations and studies have already yielded certain definite information of great importance in respect to the combating of the disease through the employment of the specific antimeningococcus serum.

Types of Meningococcus.

Biological studies of meningococci have led to their separation into two main groups, called respectively meningococci, or normal

meningococci,[1] and parameningococci (Dopter[2]). The two main groups are distinguished not by their cultural properties, but by their immunity reactions. This divergence in immunity reaction has proven of the greatest importance in perfecting the antimeningitis serum, since its action is specific and it affects those types of meningococci only for which its antibodies possess affinity.

At the outset, and before the two main groups of meningococci were clearly differentiated, the antimeningitis serum was prepared by employing a number of strains of meningococci in the immunizing process. The object, of course, was to cover ordinary variation in immunity properties of the different cultures; but with the discovery of the two types of meningococci this haphazard method no longer sufficed and it became necessary to use for purposes of immunization representatives of the two groups now designated meningococci and parameningococci. The sera now being prepared are for the most part produced by immunizing horses either with mixtures of these types of meningococci or with alternate injections of the two types.

Our recent studies indicate, however, that it does not suffice merely to employ representative normal and representative parameningococcus strains for the purpose of immunization, for while the two groups are, immunologically considered, fairly homogeneous, yet within each group there exist organisms which react weakly to the specific immune bodies produced by other strains of the same type. On the other hand, when these weakly reacting strains are themselves inoculated they evoke the formation of specific immune bodies to which they react strongly. Hence it is desirable to employ for immunization not merely one, but rather several strains of each group; and it is further desirable to change or alternate the strains as new ones are obtained which show an imperfect response to the immunity bodies already present.

The existence of different types of meningococcus was indicated early in the course of the serum treatment of meningitis through the observation that in certain cases the meningococci when brought under the influence of the antiserum failed to be affected by it. Since this was the exception, it was assumed that certain strains of meningo-

[1] Wollstein, M., *Jour. Exper. Med.*, 1914, xx, 201.

[2] Dopter, C., *Compt. rend. Soc. de biol.*, 1909, lxvii, 74.

cocci were resistant, or fast, to the antimeningitis serum. Further studies, as has been mentioned, established the fact that two great groups of meningococci could be distinguished; and our later studies indicated that even within these groups variants occur which are less subject to the action of a polyvalent antimeningitis serum than the majority of strains.

We are at present only imperfectly informed as to the relative prevalence of the different types of meningococci in given foci of epidemic meningitis. The American experiences and apparently the experiences previous to recent studies in the war zone in France and England have indicated that normal meningococci greatly preponderate in cases of epidemic meningitis. It now appears that, in certain localities at least in which epidemic meningitis prevails in France and England among the army, the proportion of cases caused by normal meningococci as contrasted with cases caused by parameningococci may be no higher than 6 : 4 (Ellis,[3] Arkwright[4]).

This consideration has definite bearing on the preparation of antimeningitis serum and emphasizes the importance of proceeding in its preparation in such a manner as to produce quantitative results in which the antibodies for the parameningococci about equal in amount those for the normal strains.

Mode of Preparation of the Serum.

At the first appearance of the present outbreak of epidemic meningitis in Great Britain, The Rockefeller Institute was no longer engaged in the preparation of the antimeningitis serum. Two circumstances led to the resumption of its manufacture. The first was the probability that the epidemics abroad would extend and the demand for the serum would exceed that available from ordinary sources of supply. Moreover, the identification of The Rockefeller Institute with the original production of the serum brought to it urgent requests for serum from several of the countries at war, with which it seemed imperative to comply. Through the assistance rendered by The Rockefeller Foundation, which has been engaged extensively in war

[3] Ellis, A. W. M., *Brit. Med. Jour.*, 1915, ii, 881.

[4] Arkwright, J. A., *Brit. Med. Jour.*, 1915, ii, 885.

relief, funds were placed at the disposal of the Institute covering the cost of production of the serum, so that it could be supplied gratis to those countries from which the demand came.

But there was a second important reason which led to the resumption of the manufacture of the serum. At the time and after the appearance of epidemic meningitis, particularly among the British recruits, the supply of serum available in England was chiefly that prepared commercially. Its use was distinctly disappointing. Realizing that in all probability the failure lay with the samples of serum available, which by reason of some fault of production or preservation were inactive, it seemed desirable to produce a serum the activity of which could be relied upon (Osler, Rolleston[5]).

In a previous communication[6] from the laboratories of the Institute a method was described by means of which the preparation of the antidysenteric serum was greatly abbreviated. The method consists in making injections into the horse of cultures or extracts of dysentery bacilli on 3 successive days, after which a period of rest is permitted. It was ascertained that the immunity response to the bacteria or bacterial products thus injected was far greater than when they were introduced at periods separated from each other by the ordinary intervals of time. Moreover, it was determined that by this rapid method an efficient polyvalent antidysenteric serum could be produced, representing both the non-acid (Shiga) and the acid (Flexner) groups of bacilli. Instead of a period of 8 or 12 months required by the usual method of immunization, an equally strong serum was produced in the short period of 8 or 12 weeks.

The problem presented, therefore, in respect to the antimeningitis serum seemed essentially similar to that encountered with respect to the antidysenteric serum. In the case of the latter, two groups of the bacilli are dealt with: first, a fixed or Shiga group, which yields a soluble toxin; and second, a fluctuating group made up of very slightly divergent types of bacilli which do not yield a soluble toxin. In the case of the meningococci there are also two groups or types, neither being perhaps altogether fixed: the ordinary or normal meningococci

[5] Discussion on the treatment of cerebrospinal meningitis, *Proc. Roy. Soc. Med., Therap. and Pharmacol. Sec.*, 1915, ix, 1.

[6] Flexner, S., and Amoss, H. L., *Jour. Exper. Med.*, 1915, xxi, 515.

which readily undergo autolysis, and the parameningococci undergoing less perfect autolysis, both yielding, however, a toxic product. Hence it could be assumed that by employing a method similar to that worked out for the antidysenteric serum, horses might be rendered immune and made to yield an efficient polyvalent antimeningococcic serum in a period of time far shorter than is required by the usual method of subcutaneous inoculation. The substances employed in both instances are the same; namely, living cultures and the extract, or autolysate, inoculated alternately. By the old method from 6 to 12 months were required to produce a meningococcic serum of high immunity value. By the new method a similar result has been achieved in from 8 to 12 weeks.

In following out this plan, a difficulty was early encountered, and one which was indeed foreseen. Two general methods of immunization of horses have been followed for the production of antimeningitis serum. In one the cultures of meningococci or the autolysate is injected subcutaneously (Flexner and Jobling[7]); in the other, the injections are made intravenously (Dopter[8]). In the first instance, no ill effects arise, aside from the occasional production of sterile abscesses. In the other, after several inoculations have been made, the horses become extremely sensitive, so that sudden death has been known to follow an injection of the culture or autolysate. This danger can, however, be prevented, as has been shown by Dopter,[9] by employing a desensitizing injection of the culture before making the full inoculation.

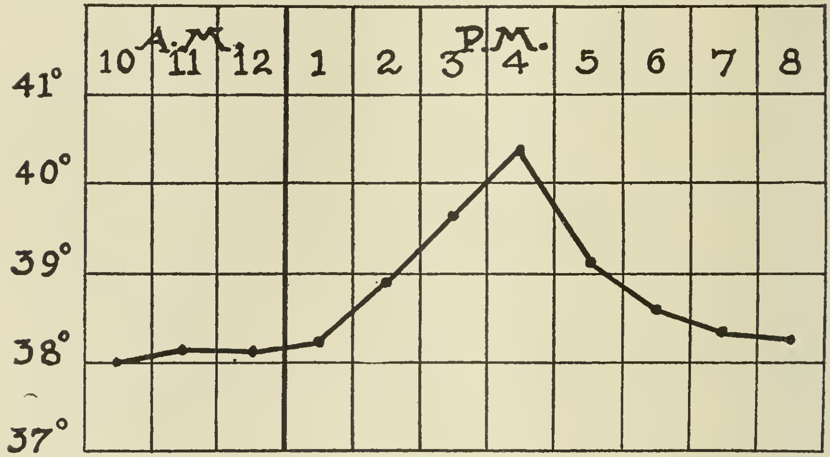
Hence the method adopted for the rapid production of the antimeningitis serum follows closely, but does not exactly reproduce, that already described for the rapid production of the antidysenteric serum. It consists in beginning with small doses of living meningococci injected daily for 3 days, followed by a period of rest of 7 days, when another series of injections is made. Thus for a 1,300 pound horse, one-twentieth of a 24 hour culture on slanted plain agar is injected on the 1st day. The material for injection is made up as follows: 2 cc. of physiological salt solution are added to a 24 hour agar slant

[7] Flexner, S., and Jobling, J. W., *Jour. Exper. Med.*, 1908, x, 141.

[8] Dopter, C., *Ann. de l'Inst. Pasteur*, 1910, xxiv, 96.

[9] Briot and Dopter, *Compt. rend. Soc. de biol.*, 1910, lxix, 174.

of meningococcus culture and the growth is suspended in it. Then 0.1 cc. of the suspension is transferred to 15 cc. of physiological salt solution and injected intravenously very slowly. The temperatures are taken hourly, beginning with the 4th hour after the injection, and continued until the temperature has reached its highest and begun to decline. 24 hours later 0.2 cc. of the suspension, and on the 3rd day 0.3 cc., that is about one-seventh of the agar slant, is used. After the lapse of 7 days, the dose given on the first of the 3 days corresponds with that given at the end of the last series; namely, in the instance in question, 0.3 cc. The temperature is again taken, and



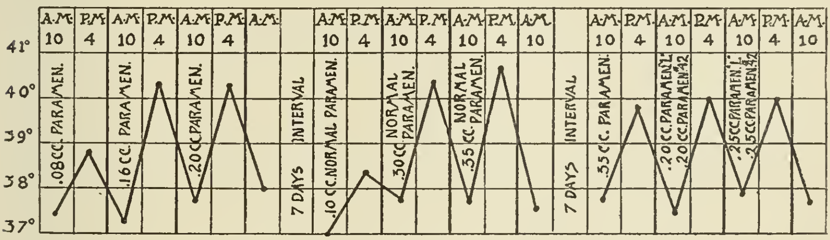
TEXT-FIG. 1. Typical febrile rise and fall following each single injection of meningococci.

if the rise does not equal 2.5–3°C. the conclusion drawn is that the dose has been too small. It is increased, therefore, for the injection 24 hours later above the usual rate of increase of 0.1 cc. according to the degree of rise of temperature. If the temperature does not fall to normal within 18 to 24 hours, the conclusion is drawn that the dose given has been too large (Text-fig. 1).

Following this plan, doses may be regulated with nicety and a maximum of reaction be obtained, we believe, with a minimum of danger. No serious effect is produced, although chills may attend the severer reactions. The greatest reaction, as a rule, is that pro-

duced by the first injection, whereas the succeeding injections on the 2nd and 3rd day tend to produce less severe reactions. Hence the increase between the second and the third injection may be larger than that between the first and second. In the second series of injections, the maximum doses have been from 0.4 to 0.45 cc., and in the third series from 0.5 to 0.6 cc. on the 3rd day. Since individual horses vary in their susceptibility, the first doses are small and the temperature curve forms the basis for adjusting the other doses (Text-fig. 2).

In Horse N only two series of injections were necessary before the typical and desired temperature curve was attained. The succeeding curves for any series agree closely with the third series in the chart.



Text-Fig. 2. Chart of Horse N showing febrile reactions and adjustment of doses in the first three series of injections.

In the succeeding series increase in the amounts injected is accomplished by the addition of new strains from time to time. For example, when the total dose is 0.6 cc. (*i.e.*, slightly more than one-third of the agar slant) it may consist of 0.2 cc. each of three strains. The largest amount of any single injection in a 1,300 pound horse has been one-fourth of each slant from seventeen different strains.

In preparing a polyvalent serum two slightly different procedures may be followed. According to one, the normal and the parameningococcus strains are inoculated within what may be designated as periods of two series; that is, the normal and the parameningococcus strains are alternated. According to the other, in which the autolysate is also employed, the period includes three series, one for the normal meningococcus, one for the parameningococcus strains, and one for the autolysate. The autolysate, in turn, is made up of equal parts

of a typical normal meningococcus and of a typical parameningococcus strain. Evidence is at hand, to be referred to below, which shows that the autolysate, at least in the sheep, excites little agglutinin formation while producing other protective principles.

Desensitization.

As stated, the horse becomes hypersensitive to the intravenous injections of the meningococci or its products apparently after the third or fourth series of injections. This reaction tends to be most severe after the first dose in each series, as might have been predicted. It was found that, acting upon the suggestion of Dopter,[9] desensitization may be effected apparently readily and certainly. Hence on the 1st day of each series about one-twentieth (later one-tenth) of a 24 hour slant of culture is injected intravenously, and 2 hours later the remainder of the dose is given. This desensitization suffices and need not be repeated on the 2nd and 3rd days, when the next doses are injected.

The danger from both brain and lung emboli must not be overlooked. Bull[10] has observed that suspensions of bacteria, introduced into the blood stream of an animal immune to the same organism, are clumped and deposited in the blood vessels of the brain, lungs, and in the spleen. If the bacteria are present in any considerable number, the clumps may occlude the cerebral and pulmonary capillaries and produce sudden death of the animal. We have found this to be true in the injection of meningococcus into normal and immune rabbits. It is possible that a part of the so called anaphylactic phenomena observed in immunization with meningococci can be explained by these facts, since the symptoms observed are similar if not identical in these two instances.

In order to avoid such a possible danger the suspensions of living meningococci are made up to a relatively larger volume, 15 to 20 cc., and introduced very slowly into the circulation.

[10] Bull, C. G., *Jour. Exper. Med.*, 1915, xxii, 466.

Strains Employed in the Immunizing Process.

In the beginning a group of five representative normal meningococcus strains and a group consisting of an equal number of parameningococcus strains were used alternately in the series of injections. The progress of the formation of immune bodies in the serum was followed by studying the agglutination, opsonization, and complement deviation reactions, with as many strains of meningococci as possible. In this manner strains not employed in the original series of inoculations may be divided into temporary lots according to their reaction with the serum. If the reactions are not within or near the zone of reaction of the strains already being used for immunization, representatives of such lots are selected and placed in the immunizing group. The remaining members of these lots are again tested later when immune bodies against the representative of the group have appeared in the serum. Should the serum now contain antibodies effective against all members of the group, it will, of course, not be necessary to include them all. Agglutination serves as the most specific index of immunological reaction except in the case of inagglutinable strains, which, in our experience, are infrequently encountered.

By the above procedure we have studied sixty-four strains of meningococci, and from this number twenty-two were selected to be added to the two original immunizing groups which consisted respectively of five normal meningococcus strains and five parameningococcus strains. For convenience in regulating the doses and for injecting we have added equally to each group.

We selected for addition to the normal group eleven of those which showed the least variation from the normal meningococcus; and the remaining eleven, exhibiting wider variations, were placed in the second group which originally consisted only of parameningococci. The strains used at present for immunizing are thirty-two, and have for convenience been divided into two lots. Lot A consisted originally of five normal and irregular strains of meningococcus, and to these have been added eleven normal and irregular strains. Lot B consisted originally of five parameningococcus strains (two from the Pasteur Institute, Paris, and three isolated in America) and to these have been added eleven parameningococcus and irregular strains exhibiting wider variation from the normal. One lot is used for three

successive injections and after a rest of 7 days the other lot is used in like manner. If care is not taken to balance the two lots, the immune bodies against one group may be developed to a greater extent than those against the other. To avoid this, the serum is tested immunologically after every second series if living organisms are injected, and after every third series if autolysate is also given. The relative doses of the two groups are regulated accordingly.

Since we have evidence that both normal meningococci and parameningococci differ among themselves in antigenic power, it is desirable to study all possible strains in the course of an epidemic in order that strains which are at variance with those being used in the production of an immune serum may be included in the immunizing groups.

This point is illustrated by the following experience with two strains. Strain NO was tested against the polyvalent serum of a horse highly immune against at least twenty normal and parameningococcus strains and found to be agglutinated in a dilution of 1 : 10. This strain, which was later found to be parameningococcus, was included in the immunizing group, and after two inoculations the agglutinins rose to 1 : 100, and after four injections to 1 : 200. When fewer strains are being used, the immune bodies may be developed more quickly. Strain Andrews, brought from England by Dr. A. Gardner Robb, when tested against a potent serum effective against ten normal meningococcus and parameningococcus strains, agglutinated in a dilution of 1 : 10. It was included in the immunizing group and after two series of injections, the agglutinating power of the serum for this strain rose to 1 : 200. We consider that the larger doses of Andrews which it was possible to employ caused the more rapid rate in the production of the agglutinins. Table I illustrates the development of agglutinins for strains relatively inagglutinable before their inclusion in the inoculation group.

TABLE 1.

Strains.	Before injection.	After 1 injection.	After 2 injections.	After 4 injections.	After 5 injections.
Andrews........	1 : 10	1 : 100	1 : 200	....	1 : 2,000
NO............	1 : 10	....	1 : 100	1 : 200	....

Autolysate.

Equal parts of toluene autolysate[11] from normal meningococcus and from parameningococcus may be injected intravenously on 3 successive days forming one series, alternating with the two series of living meningococci. The serum of horses receiving the autolysate series develops agglutinins, opsonins, or power to deviate complement less quickly than that of animals receiving only the living organisms, though power to neutralize the toxin contained in the autolysate is developed.

TABLE II.

Serum.	Agglutinins.		Opsonins.		Complement deviation.	
	Normal meningococcus.	Parameningococcus.	Normal meningococcus.	Parameningococcus.	Antigen from normal meningococcus.	Antigen from parameningococcus.
Sheep B (autolysate)...........	1 : 100	1 : 50	1 : 50	1 : 1,000	1 : 200	1 : 100
Horse L (living cultures and autolysate).......	1 : 5,000	1 : 2,000	1 : 2,000	1 : 5,000	1 : 10,000	1 : 5,000

A sheep received intravenously maximum doses of autolysate in series of 3 successive days and a rest of 7 days over a period of 9 months. Doses sufficient in size to cause marked febrile reaction and sometimes diarrhea, were administered. At the end of 9 months the serum exhibited only slight power of agglutination, opsonization, and complement deviation.

Table II shows the titer of the sheep serum compared with that of serum obtained from a horse receiving autolysate and also living meningococci.

The sheep serum when incubated with living meningococci and injected intraperitoneally into small guinea pigs was found to possess low anti-infectious value.

[11] Flexner, S., *Jour. Exper. Med.*, 1907, xx, 105.

Immunity Value of the Antiserum.

The immune bodies of the horse serum were estimated by testing its agglutinating and opsonizing power with normal and parameningococcus strains and by determining its power to fix complement in the presence of antigens made from these strains. Its anti-infectious power was determined by incubating varying amounts with one minimum lethal dose of living meningococci for 1 hour at 37°C., and injecting the mixture intraperitoneally into young guinea pigs weighing not less than 90 or more than 110 gm.

Agglutination.—Representative normal meningococci and parameningococci were selected for following the development of agglutinins.

TABLE III.

Horse L Serum.

Period.	Before injection.	1st.	2nd.	4th.	7th.*	After 6 wks. of autolysate injections.†	10th.	11th.‡	13th.	14th.
Meningococcus.......	1:30	1:60	1:80	1 : 1,000	1 : 5,000	1 : 2,000	1 : 2,000	1 : 500	1 : 1,000	1 : 2,000
Parameningococcus . .	1:10	....	1:80	1 : 200	1 : 2,000	1 : 500	1 : 1,000	1 : 1,000	1 : 1,000	1 : 2,000

* 7 periods extend over 10 weeks. 6 liters of blood were taken from the horse at this time.

† For 6 weeks after the 9th period the horse received autolysate only.

‡ Twelve new strains added. 1st period after bleeding.

Horse M Serum.

Period.	Before injection.	2nd.	4th.	7th.	10th.
Meningococcus..................	1 : 20	1 : 500	1 : 500	1 : 1,000	1 : 2,000
Parameningococcus..............	1 : 10	1 : 50*	1 : 1,000	1 : 1,000	1 : 2,000

10 periods extend over $12\frac{1}{2}$ weeks.

* This low figure shows that the injections had not been properly balanced. Too few parameningococci had been injected; accordingly larger doses were given with the result that the agglutinins increased greatly during the next periods. The highest dilution at which any of our sera agglutinated was 1 : 5,000 for both normal and parameningococci.

The reactions were made at 55°C. and read after 24 hours. Table III shows the increase of agglutinins by periods.

Opsonins.—The opsonins were estimated by the Neufeld technique. Table IV shows their development by periods.

TABLE IV.

Horse L Serum.

Period.	5th.	7th.	9th.	Interval of 6 wks. Injected with autolysate only.	10th.	13th.	15th.
Meningococcus.	1 : 500	1 : 1,000	1 : 500*		1 : 500	1 : 1,000	1 : 2,000
Parameningococcus.......	1 : 200	1 : 5,000	1 : 2,000		1 : 1,000	1 : 1,000	1 : 2,000

* Bled 6 liters just before this period.

Horse M Serum.

Period.	2nd.	7th.	11th.
Meningococcus..........................	1 : 500	1 : 200	1 : 2,000
Parameningococcus......................	1 : 200	1 : 1,000	1 : 2,000

Complement Fixation.—Tests for complement-binding bodies in the immune horse and sheep sera were made with antigens of regular normal meningococci as well as with those of irregular normal meningococcus and parameningococcus strains. The results are shown in Table V. It appears that in the serum from Horse L the power to bind complement ran fairly parallel with the power to agglutinate the meningococci.

TABLE V.

Complement Deviation by Serum L.

Period.	4th.	7th.	8th.	9th.	Interval of 6 wks. Injected with autolysate only.	10th.	11th.	12th.	13th.	14th.
Meningococcus....	1:200	1 : 1,000	1 : 1,000	1 : 2,000		1 : 1,000	1:500	1 : 1,000	1 : 1,000	1 : 1,000
Parameningococcus..	1:100	1 : 500	1 : 1,000	1 : 2,000		1 : 500	1:500	1 : 500	1 : 2,000	1 : 5,000

Complement Deviation by Serum M.

Period.	2nd.	4th.	5th.	6th.	9th.	12th.
Meningococcus	1 : 200	1 : 2,000	1 : 5,000	1 : 2,000	1 : 2,000	1 : 1,000
Parameningococcus....	1 : 200	1 : 1,000	1 : 2,000	1 : 1,000	1 : 1,000	1 : 5,000

Protective Value.—Flexner recommended the use of small guinea pigs in determining the anti-infectious power of antimeningitis serum. If young guinea pigs weighing between 90 and 110 gm. are used and the experiment is run in quadruplicate, some measure of the protective power is obtained. One minimum lethal dose of the living meningococcus is incubated with varying amounts of immune serum for 1 hour at 37°C. and then injected intraperitoneally.

Table VI shows the anti-infectious power of serum L compared with normal horse serum.

TABLE VI.

Anti-Infectious Power of Polyvalent Antimeningitis Serum.

Strain.	2 cc. of normal horse serum.	0.2 cc. of polyvalent immune serum.	0.3 cc. of polyvalent immune serum.	0.4 cc. of polyvalent immune serum.
1 m. l. d. of living meningococci.	No protection.	Protection in 1 out of 4.	Protection in 3 out of 4.	Protection in 4.
1 m. l. d. of living parameningococci.	No protection.	Protection in 3 out of 4.	Protection in 3 out of 4.	Protection in 4.

If two minimum lethal doses are used, there is little protection after 48 hours. For example, in one series the control guinea pig receiving one minimum lethal dose and those receiving two minimum lethal doses plus 0.5 cc. of serum died in 12 to 18 hours. Three out of four guinea pigs receiving two minimum lethal doses plus 0.6 cc. of serum lived between 47 and 48 hours.

Normal horse serum exerts practically no anti-infectious action either with meningococcus or with parameningococcus. Immune serum produced by the rapid method possesses a considerable degree

of anti-infectious power. About 0.4 cc. of this polyvalent serum is capable of neutralizing the infecting power of one minimum lethal dose of the living meningococcus or parameningococcus.

SUMMARY.

Potent antimeningitis serum can be safely produced in the horse by the method of three successive intravenous inoculations of living meningococci and parameningococci repeated at stated intervals.

Sudden and alarming symptoms and sudden death are avoided by employing first a desensitizing injection and then by adjusting the doses according to the febrile reaction and by making the highly diluted injections slowly.

Horses undergoing this process of immunization remain in good condition and may even gain in weight.

Specific immune bodies appear in the serum early and rise rapidly.

By inoculating alternately several strains of living meningococci and parameningococci, and the autolyzed products of each, a polyvalent serum of high titer can be produced in 8 to 12 weeks instead of in the 10 months required by the subcutaneous method.

The serum produced by this rapid method has been employed therapeutically in America, England, France, and some other countries.

It is highly desirable to isolate meningococci from many sources and test the strains against the polyvalent serum. Strains which are not agglutinated in high dilution in such a serum should be included subsequently in the lot of strains used for immunization.

THE INFLUENCE OF TYPHOID BACILLI ON THE ANTIBODIES OF NORMAL AND IMMUNE RABBITS.

By CARROLL G. BULL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 12, 1916.)

The view is held that bacterial injections cause a decrease in the antibody content of the blood of man and the lower animals.[1] This belief is largely based on the work of Wright in connection with various forms of bacterial inoculation. Wright[2] contends that every process of immunization produces a sequence of negative and positive phases, and hence that repeated inoculation may give rise to a multiple of such sequences. This phenomenon is of particular interest in connection with prophylactic typhoid vaccination and in the treatment of cases of typhoid fever with intravenous injection of typhoid bacilli or their products.

In the course of a study of native and acquired immunity of rabbits to typhoid bacilli, including the fate of the bacilli when injected intravenously,[3] certain observations were made which threw doubt on the action of the negative phase as expressed above. These incidental observations have now been extended and the complete results are reported in this paper.

Technique.

Immunization of Animals.—Rabbits were immunized to typhoid bacilli as follows: 1st day, 0.1 of an agar slant of heat-killed bacilli was injected into the peritoneum; 2nd day, 0.1 of an agar slant of heat-killed bacilli was injected into the vein; 5th day, 0.1 of an agar slant

[1] von Wassermann, A., and Sommerfeld, P., *Med. Klin.*, 1915, xi, 1307.

[2] Wright, A. E., *Brit. Med. Jour.*, 1903, i, 1069.

[3] Bull, C. G., *Jour. Exper. Med.*, 1915, xxii, 475.

of living bacilli was injected into the vein; 7th and 9th days, repetition of the 5th day treatment. 10 days after the last injection the sera gave agglutination reactions in dilutions varying from 1 : 30,000 to 1 : 200,000.

Antibodies.—The immune sera were tested for agglutinins, precipitins, opsonins, complement fixation, and bactericidins. Every reaction was not, however, carried out with the serum of each rabbit, but different groups of rabbits were often used in the different sets of experiments, as shown in the individual tables.

Agglutinins.—The fresh sera were diluted with 0.9 per cent sodium chloride solution, ranging in strength from 1 in 2 to 1 in 200,000, as the individual animals demanded, and one drop of a suspension of typhoid bacilli washed from a 24 hour agar slant (growth covering the entire surface of the slant) with 6 cc. of salt solution, was added to 1 cc. of each dilution. The quantity of bacilli was kept as uniform as possible in the various tests of the same animal's serum, since it is known that the titer of a serum depends largely upon the number of bacilli present.[4] The tubes were incubated at 37°C. for 2 hours and allowed to remain at room temperature for 2 hours before the final readings were made.

Complement Fixation.—Antigen was prepared as follows: Typhoid bacilli were grown on plain agar, washed from the agar with salt solution, and freed from particles of the medium by repeated centrifugation. The washed bacillary bodies were frozen and thawed several times, dried in vacuum over sulphuric acid, and ground to a fine powder in an agate mortar. 1 dg. of the powder was added to 100 cc. of salt solution, after which the mixture was shaken for several hours and then passed through a Berkefeld filter. The clear filtrate was used as antigen in both the complement fixation and precipitin tests. It proved highly satisfactory. From 0.05 to 0.1 cc. gave binding with 0.04 cc. of immune serum, while 0.3 cc. did not bind with normal serum. A hen-rabbit hemolytic system and guinea pig complement were employed.

Precipitins.—In testing for precipitins 0.2 cc. of the antigen was floated over 0.2 cc. of the undiluted sera and the results were read

[4] Foerster, O., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1897, xxiv, 500.

after standing for 2 hours at 37°C. The appearance of a delicate white ring at the plane of contact of serum and antigen was considered a positive reaction. The reaction was delicate and strictly specific. Filtrates from bouillon cultures of the bacteria gave non-specific reactions.

Opsonins.—The opsonizing power of the sera was determined by the Neufeld method. Equal amounts of serum dilutions, bacterial suspension, and guinea pig leukocytes were mixed and incubated for 2 hours at 37°C.; slides were then made and stained after fixation in methyl alcohol with Manson's stain. The highest dilution of serum in which the degree of phagocytosis exceeded the salt solution control was considered as indicating the opsonic titer of the serum. This test was used more extensively in examining the normal than the immune sera. As typhoid bacilli are phagocyted in salt solution, the reactions are less delicate than the other tests employed.

Bactericidins.—The bactericidal capacity of the sera for typhoid bacilli was determined as described below.[5] Bacterial suspensions of varying strengths were prepared with sterile salt solution from a 24 hour bouillon culture. The suspensions ranged from 1 in 10 to 1 in 1,000,000. 0.05 cc. of the different suspensions was put in small test-tubes, and 0.2 cc. of the serum to be tested was added to each tube. Care was taken to prevent the bacterial suspensions from touching the sides of the tubes above the level of the sera and thus to escape the action of the serum. The tubes were incubated for 1 hour, and 1 cc. of melted agar was added to each tube, and also to a set of salt solution controls. After incubation the degree of destruction can be easily determined. Sterility of the tubes or a reduction in the number of colonies to three or four was regarded as a positive reaction. A mere reduction of colonies is no doubt to be ascribed to agglutination. This test was used especially for the sera of normal rabbits and for rabbits after the first inoculation of bacilli.

[5] Wright, A. E., *Lancet*, 1901, i, 609.

EXPERIMENTAL.

Each type of experiment reported has been performed a number of times, but only a few tables illustrating the different classes will be given in detail.

In Experiment 1 (Table I) three rabbits having a high degree of immunity to typhoid bacilli were used. A sample of blood was taken from each rabbit, injections and bleedings were made, and the sera tested, as indicated in Protocols A, B, and C (Table I).

TABLE I.

Rabbit A.

Injection.	⅜ of an agar tube of typhoid bacilli.				
Time of bleeding.	Before injection.	1 hr.	5 hrs.	24 hrs.	48 hrs.
Agglutinins	50,000	50,000	50,000	50,000	50,000
Precipitins	+++	+++	+++	+++	+++
Complement fixation	+++	+++	+++	+++	+++

Rabbit B.

Injection.	⅜ of an agar tube of typhoid bacilli.						
Time of bleeding.	Before injection.	1 hr.	5 hrs.	22 hrs.	47 hrs.	70 hrs.	120 hrs.
Agglutinins	80,000	80,000	80,000	80,000	80,000	80,000	100,000
Precipitins	+++	+++	+++	+++	+++	+++	+++
Complement fixation	+++	+++	+++	+++	+++	+++	+++

Rabbit C.

Injection.	⅜ of an agar tube of typhoid bacilli.				
Time of bleeding.	Before injection.	1 hr.	5 hrs.	24 hrs.	48 hrs.
Agglutinins	30,000	30,000	30,000	30,000	30,000
Precipitins	+++	+++	+++	+++	+++
Complement fixation	+++	+++	+++	+++	+++

Experiment 1 shows definitely that an intravenous inoculation of typhoid bacilli causes no reduction in the concentration of agglutinins,

precipitins, and degree of complement fixation of the sera of rabbits up to time limits of the tests. This result was surprising. It was to be expected in view of Wright's results that an injection of the homologous bacteria would cause lowering of the various antibodies. No such reduction was detected.

The results of this experiment led to the performance of a large number of experiments of a similar nature, the number of bacilli inoculated being varied widely. It was found that the number of bacilli injected did not influence the end result. Injections were given under the skin and into the peritoneum with like results. Intravenous inoculation of the bacilli from an entire agar slant caused no reduction in the concentration of the antibodies. Lethal doses of bacilli were given and the rabbits bled while dying, but still the sera contained agglutinins, precipitins, and complement deviating antibodies in the same concentration present before the injections were made. Moreover, when sublethal doses were employed, the agglutinins were found to be increased at the 48th to the 96th hour after inoculation.

Natural and acquired antibodies show some points of difference: opsonins are thermolabile (Wright), bacteriotropins thermostabile (Neufeld); the natural agglutinins of rabbits for typhoid bacilli are thermolabile and disappear spontaneously within a few days, while the acquired agglutinins are thermostabile and persistent. Hence, it was thought possible that the bacillary injection might cause a reduction in the natural antibodies, while the acquired ones might remain uninfluenced.

Rabbits can be used advantageously for these experiments, since the sera of a large percentage contain natural opsonins, agglutinins, and bactericidal substances for typhoid bacilli. Representative protocols of experiments pertaining to the natural antibodies follow (Table II).

The protocols of Experiment 2 (Table II) represent three types of test. In one the number of bacilli injected was sufficient to cause acute intoxication and death in from 1 to 3 hours. Rabbits A^1 and B^1 fall in this group. From 1 to 2 hours after the injections the rabbits were in a state of collapse; they showed extreme relaxation, low blood pressure, heavy breathing, and, as a rule, diarrhea. Specimens of blood were taken while the rabbits were in this state or immediately

TABLE II.

Rabbit A[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 17	11.35 a.m.		1–3+	1–15+	
	11.40 a.m.	$\frac{1}{20}$ agar slant of typhoid bacilli in vein.			
	1.15 p.m.		1–3+	1–20+++	Rabbit moribund when bled and died immediately afterwards.

Rabbit B[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 17	11.25 a.m.		1–6+	1–15+	
	11.30 a.m.	Same as Rabbit A[1].			
	1 p.m.		1–6+	1–20+++	Blood taken from heart immediately after death.

Rabbit C[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 19	10 a.m.		1–20+	1–40+	
" 19	12 m.	$\frac{1}{30}$ agar slant of typhoid bacilli in vein.			
" 19	2 p.m.		1–20+	1–40++	Heavy breathing; collapse; diarrhea.
" 19	5 p.m.		1–20+	1–40+++	Rabbit very weak.
" 20	10 a.m.		1–20+	1–90+	
" 21	9.30 a.m.		1–24++	1–90+	
" 22	8 a.m.		1–50+	1–300+	Rabbit found dead, and unclotted blood removed from heart. Blood sterile.

TABLE II—*Continued.*

Rabbit D^1.

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 19	10 a.m.		1–12+	1–40+	
" 19	12 m.	Same as Rabbit C^1.			
" 19	2 p.m.		1–12+	1–40+	Collapse; diarrhea.
" 19	5 p.m.		1–12+++	1–60+	
" 20	9 a.m.		1–12+++	1–60+	
" 21	10 a.m.		1–16+++	1–80+	
" 21	11.45 a.m.	Rabbit died. Two agar slants and one tube of bouillon were each inoculated with one loop of heart's blood. One agar tube had two colonies, one was sterile; the bouillon gave a culture.			

Rabbit E^1.

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 19	10 a.m.		1–12+	1–30+	
" 19	10.20 a.m.	$\frac{1}{40}$ agar slant of typhoid bacilli in vein.			
" 19	12.20 p.m.		1–12+	1–30+	Rabbit flaccid.
" 19	3.20 p.m.		1–12+	1–40+	
" 20	10 a.m.		1–12+	1–40+	
" 21	9 a.m.		1–12+	1–50+	
" 22	10 a.m.		1–24+	1–70+	
" 23	10 a.m.		1–160+	1–200+	
" 24	10 a.m.		1–160++	1–500+	
" 27	12 m.		1–300+	1–5,000+	
" 27	12.30 p.m.	$\frac{1}{20}$ agar slant of typhoid bacilli in vein.			
" 27	2.30 p.m.		1–300+	1–5,000+	
" 27	6 p.m.		1–300+	1–5,000+	
" 28	9 a.m.		1–300+	1–5,000++	
" 29	9 a.m.		1–400+	1–20,000+	Rabbit in good condition.

TABLE II—*Concluded.*

Rabbit F[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 19	2 p.m.		1–12+++	1–40+	
	2.45 p.m.	$\frac{1}{40}$ agar slant of typhoid bacilli in vein.			
" 19	5 p.m.		1–16+	1–60+	
" 20	8.30 a.m.		1–12+	1–60+	
" 21	9 a.m.		1–20+	1–120+	
" 22	9 a.m.		1–40++	1–280+	
" 22	10.15 a.m.	$\frac{1}{20}$ agar slant of typhoid bacilli in vein.			
" 22	12.15 p.m.		1–40++	1–280+	
" 22	3.15 p.m.		1–40++	1–280+	
" 24	9 a.m.		1–60++++	1–400+	Rabbit in good condition.

after death and compared for opsonins and agglutinins with the blood taken before the injection. The sera obtained subsequent to the administration of the bacilli agglutinated the bacilli more actively and in higher dilutions than those taken before the bacilli were injected. A few exceptions were noted, but a lowering of the agglutinating strength never occurred. An increase in opsonizing power was not observed; no difference could be detected between the sera in this respect before and after inoculation. This fact is probably due to the circumstance that the opsonic reaction is less delicate than the agglutination test.

In another series, represented by Rabbits C[1] and D[1], fewer bacilli were injected and the rabbits succumbed in from 1 to 3 days to a more chronic intoxication. The chief symptoms noted were emaciation, anemia, and loss of appetite. At autopsy, the blood was sterile or contained very few bacilli. The sera frequently showed an initial increase in agglutinating power a few hours after the inoculation, and then a steady increase in from 24 to 48 hours and continuing up to the time of death.

In the case of Rabbit C^1, the serum obtained before the bacilli were given showed agglutination in a dilution of 1 in 40, while at about the 72nd hour the reaction was present in a dilution of 1 : 300. This rabbit was markedly anemic; no increase in opsonizing power was detected until 24 or 48 hours after the inoculation, at which time an increase was evident.

A third group is represented by Rabbits E^1 and F^1. Here repeated sublethal inoculations were made. The first injections were followed by results similar to those in Rabbits C^1 and D^1; namely, an initial increase in agglutinins and a gradual rise in opsonins and agglutinins from the 24th to the 48th hour on. The second injections were followed by a marked increase in both opsonins and agglutinins, beginning at the 24th to the 48th hour without any intervening negative phase.

A separate group of rabbits was used for the bactericidal tests, as it was often difficult to obtain enough blood from one rabbit at the various bleedings for all the tests. Protocols representing these experiments are tabulated in Table III.

The results of Experiment 3 (Table III) show a marked increase in the bactericidal power of the sera obtained from the blood taken at short and frequent intervals after the bacilli were inoculated. Five- or tenfold increase was noted 3 hours after the injection, and the increase was still greater at the expiration of 24 hours. The control, Rabbit C^2, was used to determine whether the variations observed with the sera from the inoculated rabbits were due to the different ages of the sera at the time of the tests, for they were from 1 to 24 hours old when tested. The results obtained suggest rather that remaining on the clot over night increases the bactericidal power of the sera. At least it may be said that the age of the sera was not the cause of the effects obtained. It is also evident that the sera of different rabbits vary considerably in bactericidal as they do in opsonic and agglutinating capacity. The increase in bactericidal power is not so pronounced when fewer bacilli are inoculated; $\frac{1}{80}$ or $\frac{1}{40}$ of an agar slant caused only a slight increase. It appears that a severe intoxication is necessary to cause this mobilization of the bactericidal substances.

TABLE III.

Rabbit A^2.

Injection.	Time of bleeding and injection.	Serum.	No. of bacilli killed.
		cc.	
$\frac{1}{20}$ agar slant in vein.	Before injection.	0.2	100
	3 hrs. after "	0.2	10,000
	5 " " "	0.2	10,000
	24 " " "	0.2	1,000,000

Rabbit B^2.

Injection.	Time of bleeding and injection.	Serum.	No. of bacilli killed.
		cc.	
$\frac{1}{20}$ agar slant in vein.	Before injection.	0.2	10,000
	3 hrs. after "	0.2	50,000
	5 " " "	0.2	1,000,000
	This rabbit died $5\frac{1}{4}$ hrs. after injection.		

Rabbit C^2.

Injections.	Time of bleeding and injection.	Serum.	No. of bacilli killed.
		cc.	
No injection.	Blood taken when the other rabbits were bled and tested at the same time.		
	Before injection.	0.2	1,000,000
	3 hrs. after "	0.2	1,000,000
	5 " " "	0.2	100,000
	24 " " "	0.2	100,000

DISCUSSION.

The data presented in this paper show conclusively that inoculation of typhoid bacilli causes no reduction in the concentrations of the natural or acquired antibodies present within the blood. Notwithstanding the fact that a decrease was expected, it was found that neither subcutaneous nor intraperitoneal nor intravenous injections sufficed to bring about the negative phase of Wright. It might have been supposed that large intravenous injections would remove the anti-

bodies from the blood as large quantities of cultures do from the serum *in vitro;* but no such effect was detected. This result may receive partial explanation from the fact that bacteria are quickly removed from the circulation through agglutination and accumulation in the organs and tissues,[6] but obviously the quantity of agglutinin thus engaged affects but little the whole quantity present.

Attention is especially drawn to the fact not only of the absence of the negative phase, but to the actual increase of the natural antibodies following the intravenous inoculations of the bacilli. The time required—from 1 to 3 hours—for the increase to become manifest is obviously too short for actual production to occur; the only alternative seems, therefore, a mobilization of preformed antibodies from the internal organs. Just what the source of the mobilized antibodies is has not been determined. The inoculations cause extensive destruction of leukocytes; but whether their disintegration liberates the antibodies cannot be stated. It is convenient to imagine that the beneficial effects said to follow the treatment of typhoid fever by means of the intravenous injection of specially prepared typhoid bacilli may be ascribed to this rapid mobilization of antibodies.

SUMMARY.

The subcutaneous, intraperitoneal, or intravenous inoculation of cultures of typhoid bacilli did not cause, as far as could be determined, a decrease in the antibody content of the blood serum of the rabbit.

On the other hand, the intravenous inoculation of typhoid bacilli causes a rapid mobilization of normal antibodies, thus increasing their concentration in the blood, to be followed somewhat later, as in the other forms of inoculation, by the production of so called acquired antibodies.

No such condition as the negative phase of Wright was discovered, although especially looked for in the experiments.

I am indebted to Miss Ida W. Pritchett for technical assistance in connection with the above study.

[6] Bull, C. G., *Jour. Exper. Med.*, 1915, xxii, 475.

KLOSSIELLA INFECTION OF THE GUINEA PIG.

By LOUISE PEARCE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 63 TO 70.

(Received for publication, January 31, 1916.)

In studying the lesions produced by arsenical compounds in the kidneys of different species of animals, certain parasites were observed in the renal tubules of guinea pigs, which strikingly resembled the coccidium, *Klossiella muris*, observed and described by Smith and Johnson[1,2] in the kidneys of the mouse. As we believed the guinea pig to be relatively free from spontaneous renal lesions, a search through the literature was made to ascertain if renal parasites of the guinea pig had been previously described. At the same time the study of a large number of guinea pig kidneys was undertaken, in order to determine the relative frequency of this parasite as well as to obtain information concerning the lesions which tend to be associated with it.

Seidelin[3] in 1914 describes a parasite which occurred in the kidneys of two guinea pigs from Nigeria, and which bears such strong resemblance to *Klossiella muris* in some of its stages that he considers that both parasites "must be regarded as belonging to one and the same genus, whilst the question of their specific identity or otherwise cannot at the present time be finally decided." He further states that the parasite does not appear to be a common one in West African guinea pigs, as he found only one case among about twenty guinea pigs of the Yaba series. Later he found identical parasites in sections of a kidney of another guinea pig from the same locality. He found no similar instance in guinea pigs procured in Liverpool.

[1] Smith, T., *Jour. Comp. Med. and Surg.*, 1889, x, 211.
[2] Smith, T., and Johnson, H. P., *Jour. Exper. Med.*, 1914, vi, 303.
[3] Seidelin, H., *Ann. Trop. Med. and Parasit.*, 1914–15, viii, 553.

Seidelin describes some of the stages of the parasite detected by him and suggests a probable life cycle. There is undoubtedly a close resemblance between this parasite and *Klossiella muris*, and again between both these parasites and the one about to be described. On the other hand, the question of the identity of Seidelin's parasite and the one we have observed will be discussed later on in this paper. There are certainly points of similarity between the two, as well as some points of difference.

It may be stated here that we have found the infection to be by no means uncommon in kidneys of guinea pigs. In sixty guinea pigs examined for renal parasites, twelve were found to be infected. In other words, in our experiments with guinea pigs we found that we must reckon upon at least 20 per cent of the animals from our sources being infected with this renal parasite.

The material examined consisted of kidney sections of sixty adult guinea pigs from two sources, one in Philadelphia, the other in New Brunswick, New Jersey. These pigs were either perfectly normal animals or were animals which had been used for toxicological experiments with various arsenical compounds and had survived the use of the drug not more than 24 hours. This arbitrary time limit was set in the choice of material in order that any chronic pathological process observed might not be confused with the drug action, since within this time (24 hours) no extensive proliferative change in the kidney can be attributed to the action of these compounds. The kidney tissue was fixed in Zenker's fluid and imbedded in paraffin. Sections were stained with hematoxylin and eosin, methylene blue and eosin, and Giemsa's stain. In addition, frozen sections were made from tissue fixed in 10 per cent formalin and stained with hematoxylin and eosin.

Description of the Parasite.

Various stages of the parasite have been seen in the kidneys of guinea pigs and in order to facilitate an interpretation of its probable life cycle, a certain sequence of description will be observed, beginning with the stage or form most frequently encountered.

Sporoblast Cycle.—The form most frequently seen is a small round or ovoid body measuring 7 to 8 μ in diameter. It stains a very light

bluish pink with hematoxylin and eosin, and contains numerous small dark blue masses of chromatin scattered through the cytoplasm in no definite arrangement (Figs. 1 and 2). The cytoplasm is somewhat granular and slightly, if at all refractile. The chromatin is of two sizes, larger irregular masses and tiny pin points, both taking a dark blue stain with hematoxylin and methylene blue. Usually several of these parasites are found free in the lumen of the renal tubules and sometimes the lumen appears to be completely blocked with them. They are most frequently seen in the straight tubules of the inner half of the cortex, but are also found somewhat less numerously in the convoluted tubules and in the tubules of the upper medulla. Occasionally one or two may be found in the capsular space of the glomerulus. This stage of the parasite is apparently very similar to the so called "daughter sporoblast" stage of *Klossiella muris* described by Smith and Johnson.

In certain sections it has been possible to make out a confining cell membrane surrounding several of these bodies, as in Fig. 2. The membrane is all that remains of the epithelial cell that originally contained the developing parasites. As they grow and increase in size the host cell must necessarily enlarge until it becomes flask-shaped and bulges out into the lumen of the tubule. The parasites occupy the broad distal portion of the containing cell and the nucleus is usually crowded inwards and often to one corner of the cell. It frequently appears flattened, compressed, or shrunken, and eventually it disappears, apparently just preceding or about the time of rupture of the cell. The cytoplasm of the host cell gradually becomes more and more scanty and granular and finally all that remains of the cell is its enormously distended cell wall, which is ruptured by the escaping parasites. It is somewhat difficult to determine just how many parasites develop in the epithelial cell, as we have had no opportunity of examining any material except cross sections of kidney tissue. However, there are probably at least ten to twelve.

Each of these parasites next undergoes a division or segmentation into eight to twelve bodies (Fig. 3). These bodies are falciform or banana-shaped and are extremely small, measuring 1 by 4 to 5 μ, and are surrounded by a distinct membrane. Their cytoplasm is clear and non-granular, pink (hematoxylin and eosin, and Giemsa's

stain), and each body contains a dark blue dot of chromatin. We believe that these tiny fusiform bodies are similar to the form designated as the sporozoites of *Klossiella muris*. The further history of these bodies is entirely conjectural and will be referred to later in the discussion.

Not uncommonly one will find in heavily infected kidneys a form (Fig. 4) which is evidently the precursor of the two stages just described. This form is comparatively large, and may measure 17 by 22 μ; it has the same appearance and staining characteristics as the smaller more usual forms first described (Figs. 1 and 2). A breaking up of this large mass into several constituent smaller bodies results in a picture similar to Fig. 2. In studying various infected kidneys, we have seen several microscopic fields in which there were all gradations of this division (Fig. 6), so that we are inclined to regard this large parasitic mass as corresponding to the so called "mother sporoblast" stage of *Klossiella muris.*

Ring Form.—In several of the sections of heavily infected kidneys we have seen a stage of the parasite which we have called the "ring," or "annular" form (Figs. 7 and 8). It is always within an epithelial cell and the cell itself is enlarged and may protrude into the lumen of the tubule to the extent of nearly occluding it, as in Fig. 8. This form measures 18 to 20 μ in diameter and consists of a series of twelve to eighteen definite divisions or segments arranged in a circle or ring. Each division is extremely small, ovoid in shape, and measures 3 by 5 to 7 μ. They are smaller than the more frequently encountered forms seen in Figs. 1, 2, 3, and 4 (the daughter sporoblasts), and in appearance are totally different. The cytoplasm is refractile and stains practically not at all, or at most a very pale pink with hematoxylin and eosin, and Giemsa's stain. Each segment contains one or two chromatin dots. In some instances the two chromatin dots appear to be fusing together; in others they are distinctly separate from one another. In other instances the one chromatin dot is elliptical in shape, and it is possible that the finding of two chromatin dots is due to the level of the section. In the center of the ring form is a small amount of a granular refractile pale pink staining material which is apparently a residuum of the cytoplasm of the surrounding ring segments (Figs. 7 and 8).

In Fig. 8 there is a typical ring body and another intracellular form which we think is also a ring body seen from the outside. In other words, a ring body is a section through such a hollow sphere as is seen in Figs. 9 and 10. Here there are eighteen small segments or divisions, each of which is similar in appearance and staining reactions to the segments of the ring form.

The ring segments are not only considerably larger than the final divisions of the sporoblast cycle, the sporozoites (Fig. 5), but are also of a totally different shape and appearance. Moreover, the immediate precursor stage, the ring form, is still enclosed in an epithelial cell, while the precursor stage of the falciform bodies shown in Fig. 5 is not necessarily intracellular. Indeed, when the division into the tiny falciform bodies or sporozoites occurs, the daughter sporoblasts are probably always extracellular and free in the lumen of the tubule. In addition, the sporozoites are contained in a very distinct spore, having a definite membrane, while there is no such structure surrounding the segments of the ring form. When the ring divides, the resulting segments are all apparently set free in the remains of the epithelial host cell and eventually in the lumen of the tubule when this cell ruptures. The ring segments seem to be identical with still another stage of the parasite about to be described and may represent its earliest and youngest form.

Hyaline Forms.—Figs. 11 to 16 illustrate a single stage of the parasite which from its appearance and staining character is evidently one of the segments of the ring form. It is very small, measuring from 5 to 8 μ in length and 3 to 5 μ in breadth, and is oval or ovoid in shape. The cytoplasm is non-granular, extremely refractile, hyaline, and stains pink with hematoxylin and eosin, or Giemsa's stain. It contains a relatively large clear-cut mass of dark blue staining chromatin placed towards one end of the parasite. At the opposite pole a small clear non-staining area may be seen, as in Fig. 12. In the section from which Fig. 11 was taken this clear area is visible, but it is very small and barely shows in the photograph. This stage measures 3 by 5.5 to 7 μ. It may be extra- or intracellular, as shown in the illustrations. In Fig. 12 it apparently is about to enter an epithelial cell of a renal tubule. In Fig. 11 it is clearly intracellular and surrounded by a vacuole. The nucleus of the host

cell is immediately below the parasite. The epithelial cell opposite the parasite on the other side of the renal tubule contains an ovoid inclusion which is probably a similar parasitic body, although the mass of chromatin is not seen in the section. The cytoplasm of the two cellular inclusions is identical.

In Fig. 13 another intracellular parasite is seen and here the chromatin has divided into two distinct parts. The parasite itself measures 5 by 7 μ. The host cell has become enormously enlarged and protrudes into the lumen of the tubule. Fig. 14 shows a further division of the chromatin into four distinct segments. This parasite is also intracellular, but in order to bring the chromatin into focus for the photograph, the epithelial cell outlines are not seen. Just below this parasite is another smaller intracellular form similar to those shown in Figs. 11 and 12, with only one chromatin mass.

Schizogonic Cycle.—Fig. 17 illustrates a spherical form which we are inclined to believe represents the schizogony of the parasite. It it comparatively large—measuring 22 μ in diameter, and is composed of a large number (thirty to forty) of tiny fusiform bodies or merozoites enclosed in a rather indistinct and apparently very thin membrane. It does not appear to be enclosed in an epithelial cell, but is extracellular and free in the lumen of the renal tubule. The merozoites in longitudinal section measure 1.5 by 7 to 8 μ. Their cytoplasm stains pink with hematoxylin and eosin, and each contains a tiny dot of chromatin. These small falciform bodies are extremely similar to the sporozoites described above. The general appearance of this large form suggests its similarity, if not identity with the glomerular body of *Klossiella muris*, which Smith and Johnson interpret as the schizogony of the mouse parasite. We have not found this form in the capsular space of the glomeruli but in the convoluted tubules of the first order. It is not a common stage and we have found it in only one kidney.

Pathological Changes in the Kidneys of Guinea Pigs Associated with the Parasitic Infection.

The kidneys of guinea pigs infected with the parasite show certain pathological changes of a chronic nature which we are inclined to attribute to the presence of the organism. They have been found

in all cases in which the parasite has been seen, and in several instances where these lesions were observed the infection was very light, and the parasite was found only after a thorough search.

The lesions which we think are caused by the parasite consist in an irregular accumulation of fibroblasts and small round cells about the base of some of the glomeruli. Both the distribution and arrangement of this infiltration are very irregular (Figs. 18 to 21). In a single microscopic field one or two glomeruli may be affected in this manner, while the adjacent glomeruli are normal in appearance. Moreover, the extent of the infiltration varies considerably. Some glomeruli have only a slight accumulation of round cells about their base, others are almost obliterated, as in Figs. 18, 20, and 21. Usually there seem to be relatively more cells of the small round mononuclear type than fibroblasts. The fibroblasts themselves are apparently not young cells.

These cellular accumulations seem to be fairly well confined to the immediate vicinity of the glomeruli. In certain instances, however, the round cells and fibroblasts extend outward to a limited degree into the labyrinthine tissue and along the medullary rays between the tubules, but in these cases the connection between this extension and the accumulation of similar cells about the neighboring glomeruli can be easily traced.

There seems to be no reaction of the kidney tissue in the immediate vicinity of the parasite itself, that is, in the inner half of the cortex where we have found the parasite to be most numerous. Here one may see half a dozen consecutive tubules filled with parasites and eight or ten epithelial cells containing ring forms, yet there is apparently no abnormality in the immediate interstitial connective tissue. The glomeruli, however, just above these infected tubules show a more or less extensive infiltration of round cells and fibroblasts. The portion of medulla just below the infected tubule shows no appreciable change. No gross changes in the kidneys referable to the renal parasite have been noted.

DISCUSSION.

The description of the various stages of the parasite found in the kidney of the guinea pig has been arranged so as to relate, tentatively at least, those forms which seem to belong to the same cycles

of development. Certainly two different cycles of development have been observed. The exact interpretation of the cycles must be more or less hypothetical, since there is an obvious lack of knowledge of all the stages in the evolution of the parasite.

The first cycle, described under the sporozoites is similar to the so called sporoblast stage of *Klossiella muris* and is evidently similar to the stage described by Seidelin. However, we have never seen more than twelve daughter sporoblasts, and usually only eight to ten resulting from the first division of the mother or pan sporoblast form, while Seidelin gives sixteen to twenty as the probable number. This discrepancy, if the two parasites are identical, can probably only be settled by the study of fresh material or an extensive series of sections. In the second division, into sporozoites, we have never observed more than twelve, each sporozoite measuring 1 by 4 to 5 μ. Seidelin describes thirty sporozoites, each measuring 1.5 by 8 μ. If this difference in the number of sporozoites continues to hold after the study of fresh tissue, we shall be inclined to believe that the two parasites belong to different species.

That this cycle of the parasitic development represents the sporoblast phase is strongly suggested by the fact that the sporozoites are confined in what is apparently a typical spore. We have never seen these spore-like bodies breaking up in the lumen of the kidney tubules, and it is probable that the spores containing the sporozoites are excreted in the urine, which is afterwards swallowed by the same or other guinea pigs, and that the spore membrane is digested away by the gastric juice, thus freeing the sporozoites. The extremely small size and fusiform shape of these tiny bodies would doubtless enable them to pass through the gastric or intestinal mucosa into the blood stream and so into the kidney. On the other hand, we have never seen any sporozoites in the glomerular tuft or capsular space.

The ring forms are not numerous, but they are easily found in heavily infected kidneys. They differ markedly from any stages of the sporoblast cycle and do not appear to be an integral part of it. It is easy to differentiate between a mother sporoblast, for instance, and a ring form seen from the outside, or cut on a tangent, so that the annular appearance is not seen. Moreover, the resulting division or segments of the two forms are apparently very dissimilar. The

sporozoites are slender fusiform bodies, 1 by 4 μ, with a tiny dot of chromatin; the ring segments are oval or ovoid, 3 by 5 to 7 μ, with a relatively large mass of chromatin. The ring segments are apparently identical with the small hyaline forms seen free in the lumen of the renal tubules or in the epithelial cells, and there is nothing in the nature of a spore membrane about the dividing ring to prevent their escape into the tubules when the ring completely segments. The final interpretation is, of course, one of conjecture only, but the cycle is strongly suggestive of a sexual phase. The comparatively large number of ring segments or hyaline forms suggests further that they may be the microgametes, and that the ring form may be the microgametocyte. We have not seen any bodies which we could interpret as macrogametes, or any process of fertilization or conjugation, unless Fig. 16 represents this phase.

Smith and Johnson describe in the sporoblast stage of *Klossiella muris* a budding process in which the chromatin occupies the periphery of the budding masses. One might think that the ring form is simply a cross section of some of these buds of the mother sporoblast. But the ring form possesses not only a different type of protoplasm from the mother or daughter sporoblast, but in addition its segments are totally unlike either the daughter sporoblasts on the one hand or the sporozoites on the other.

The asexual or schizogonic cycle is apparently represented by the large extracellular segmenting form seen in Fig. 17. It resembles the glomerular body described by Smith and Johnson and interpreted by them as the schizogonic form. We have not seen it in the glomerular space, but only in the convoluted tubules and its scarcity may be tentatively explained on the assumption that such a stage is present only in early and light infections. Later, apparently, the sporoblast cycle may supersede the schizogony, for this cycle only has been detected in our specimens of heavily infected kidneys. Its extremely large size and the great number of its segments or divisions preclude its belonging to either the sporoblast or ring cycles. Moreover, its situation free in the convoluted tubules is one of the locations where one would expect to find the development of such a stage, if, as we have already suggested, the infecting sporozoites find their way into the kidney by the blood stream. The merozoites or

segments of this large body are similar to the sporozoites, the final divisions of the sporoblast cycle. The conspicuous difference between the two stages of segmentation, aside from the difference in their number, is that the sporozoites are enclosed in a definite spore membrane, while the existence of a membrane surrounding the merozoite is problematical. In the specimens we have seen there is a very indistinct membrane, which, as in Fig. 17, is apparently ruptured, allowing the escape of the merozoites into the renal tubules.

Seidelin found no glomerular bodies as described by Smith and Johnson for *Klossiella muris*, but he considers that some of his tubular forms appear identical with the glomerular forms depicted by them. Unfortunately, Seidelin gives no illustration of this particular tubular form, so that we are unable to compare satisfactorily the two apparently corresponding stages. Seidelin is inclined to the opinion that these tubular forms represent the schizogony. Smith and Johnson think that their glomerular body is the schizogony for *Klosiella muris* and that the tubular forms are stages in the sporoblast cycle.

The pathological changes in the kidney of guinea pigs which we associate with the presence of this parasite are slight but definite and consist of an irregular accumulation of round cells and fibroblasts about some of the glomeruli. There is but slight involvement of the labyrinthine tissue adjacent to the glomeruli and apparently none at all in the lower or inner half of the cortex where the majority of the parasites are found. This may be due to the fact that the infecting sporozoites enter the kidney by way of the glomerular capillaries and that here the most serious injury to the kidney occurs, with a subsequent infiltration of round cells and fibroblasts.

SUMMARY.

We have found in the kidneys of twelve supposedly normal guinea pigs, coming from Pennsylvania and New Jersey, a parasite that closely resembles in some of its phases *Klossiella muris*, described by Smith and Johnson, and the renal parasite of two West African guinea pigs, described by Seidelin.

The forms most commonly found by us and described as the sporoblast cycle, are evidently similar to those described by Smith

and Johnson and by Seidelin. There are certain discrepancies of measurement between the parasite described by Seidelin and the one here described, but the most important difference between the two is the different number of sporozoites resulting from a final division of the daughter sporoblasts. Seidelin has found thirty sporozoites; we have found from eight to twelve, while the usual number is eight. Further, we have found a ring form which is unlike any of the stages in either the sporoblast or schizogonic cycle, and which we interpret tentatively as the male element or microgamete. In addition, we have found a tubular form which resembles the glomerular body of *Klossiella muris* and which we think is the schizogonic phase of this parasite.

EXPLANATION OF PLATES.

The illustrations are all from untouched microphotographs except Fig. 8, which is a drawing of an actual microscopic field. All the specimens except Fig. 17 are from Zenker fixed tissue.

Plate 63.

Fig. 1. Renal tubules showing a heavy parasitic infection in different stages, the majority being daughter sporoblasts. Hematoxylin and eosin. × 675.

Fig. 2. Eight daughter sporoblasts enclosed in an epithelial cell of a convoluted tubule. Hematoxylin and eosin. × 1,000.

Fig. 3. Three spores containing seven to nine sporozoites. Note the distinct spore membrane. Hematoxylin and eosin. × 1,000.

Plate 64.

Fig. 4. Mother sporoblast enclosed in an epithelial cell. The cell outline is not distinct in the photograph. Giemsa's stain. × 1,000.

Fig. 5. Glomerulus containing a single parasite, probably a young mother sporoblast in the capsular space. Hematoxylin and eosin. × 1,000.

Fig. 6. Three greatly enlarged epithelial cells, two containing mother sporoblasts, the third in the center containing eleven daughter sporoblasts. Hematoxylin and eosin. × 1,000.

Plate 65.

Fig. 7. Two ring forms in adjoining tubules, each enclosed in an enlarged epithelial cell. The ring to the right shows ten divisions. Hematoxylin and eosin. × 1,000.

Fig. 8. Two ring forms in distended epithelial cells; the lower form is seen from the outside. Hematoxylin and eosin. × 1,000.

PLATE 66.

FIG. 9. Two ring bodies and several daughter sporoblasts. The ring body on the left is a tangential section through the edge of a hollow sphere. Methylene blue and eosin. $\times$ 1,000.

FIG. 10. The same field as in Fig. 9, but at a lower level, showing that the so called ring body is a section through a hollow sphere. Methylene blue and eosin. $\times$ 1,000.

PLATE 67.

FIG. 11. Two intracellular hyaline forms, the one to the right showing chromatin. Hematoxylin and eosin. $\times$ 1,000.

FIG. 12. An extracellular spindle-shaped hyaline body showing the chromatin mass at one pole and a clear non-staining area at the opposite pole. One extremity of this hyaline form is in the protoplasm of an epithelial cell. Hematoxylin and eosin. $\times$ 1,000.

FIG. 13. An intracellular hyaline form in an enlarged epithelial cell. The chromatin has divided into two masses. The parasite is in a vacuole in the host cell. Hematoxylin and eosin. $\times$ 1,000.

FIG. 14. Two intracellular hyaline forms; the chromatin of the upper parasite has divided into four masses. Giemsa's stain. $\times$ 1,000.

PLATE 68.

FIG. 15. Two small intracellular hyaline forms. Giemsa's stain. $\times$ 1,000.

FIG. 16. Two intracellular hyaline bodies. The larger one shows two elongated chromatin masses. The smaller parasite to the left is at a level which shows no chromatin. Giemsa's stain. $\times$ 1,000.

FIG. 17. A schizogonic form showing thirty to forty merozoites. This body is free in the tubule and has no definite membrane surrounding it. Taken from a frozen section fixed in 10 per cent formalin. Hematoxylin and eosin. $\times$ 1,000.

PLATE 69.

FIG. 18. Irregular cellular infiltrations, especially about the base of some of the glomeruli. Hematoxylin and eosin. $\times$ 125.

FIG. 19. Three glomeruli showing very slight cellular infiltration about the base. Two glomeruli show no such infiltration. Hematoxylin and eosin. $\times$ 210.

PLATE 70.

FIG. 20. The two lower glomeruli show a slight irregular accumulation of round cells and fibroblasts. The upper glomeruli are normal. Hematoxylin and eosin. $\times$ 210.

FIG. 21. The two glomeruli to the right show a fairly extensive accumulation of round cells and fibroblasts with an irregular extension into the labyrinth. The glomeruli to the left are practically normal. Hematoxylin and eosin. $\times$ 210.

CHEMOPATHOLOGICAL STUDIES WITH COMPOUNDS OF ARSENIC.

IV. The Character and Distribution of Renal Injury Produced by Arsenicals as Indicated by the Processes of Repair.

By LOUISE PEARCE, M.D., and WADE H. BROWN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 71 to 76.

(Received for publication, January 31, 1916.)

In previous papers[1] attention was directed to some of the striking differences in the gross and histological changes produced in the kidneys of dogs by lethal doses of certain compounds of arsenic. Upon the basis of these observations it was pointed out that arsenicals could produce a so called tubular as well as a vascular injury of the kidney and that innumerable combinations of these two fundamental forms of tissue injury were obtainable through the use of arsenical compounds of different chemical constitution.

In order to obtain additional information as to the character and location of the specific renal injury produced by these arsenicals, as well as a knowledge of the subsequent processes of organic repair, we have extended our experiments to a study of tissue changes in the kidneys of animals given sublethal doses of these substances.

EXPERIMENTAL.

In the following experiments guinea pigs were used, as in our experience the kidneys of these animals are as free from spontaneous lesions as those of any animal available for the purpose. However, it should be mentioned in this connection that in the kidneys of some guinea pigs we have found certain pathological changes that seem to be associ-

[1] Pearce, L., and Brown, W. H., *Jour. Exper. Med.*, 1915, xxii, 517, 525.

ated with the presence of a renal parasite.[2] The relative importance of this finding in interpreting the various lesions in the processes of repair will be considered in the discussion.

In our previous experiments[1] dogs were used because the comparatively large size of their kidneys showed gross changes advantageously. However, as we stated then, the histological changes were controlled by experiments carried out with guinea pigs. The gross and microscopic changes in the kidneys of both dogs and guinea pigs following lethal and sublethal doses of these arsenicals were practically identical with but one exception, that of arsenophenylglycine, to which we shall refer at greater length in discussing the results obtained with this compound.

Sterile solutions of arsenious acid, arsenic acid, sodium cacodylate, salvarsan, neosalvarsan, arsacetin, arsenophenylglycine, and atoxyl were injected intraperitoneally into adult male guinea pigs. With each compound, one sublethal dose was given and the animal killed after 2 to 5 days. In addition, with the exception of the salvarsan experiment, other guinea pigs were given two to five small doses of the drug and killed in 3 to 19 days. In a few instances the animal died within 24 hours after receiving a repeated dose. In these guinea pigs an acute lesion complicates the one of longer duration. We were guided in the determination of the size of the sublethal dose by our knowledge of the character and extent of the renal injury caused by lethal amounts of the arsenical in question. We tried always to inject a non-fatal dose, yet one large enough to cause definite renal injury.

Arsenious Acid.

The acute injury to the kidney produced by large doses of arsenious acid is primarily one of congestion and dilatation of the blood vessels with hemorrhage; the tubular epithelium is degenerated with occasional slight cell necrosis and usually there is a voluminous granular precipitate in the glomerular capsule and the tubules. The restoration to normal after a moderate dose of arsenious acid is very rapid and there is but slight evidence of the previous injury (Fig. 1). This consists of large, rather irregular glomeruli with somewhat

[2] Pearce, L., *Jour. Exper. Med.*, 1916, xxiii, 431.

shrunken tufts and a distinct although slight increase in tuft nuclei. About the base of the glomeruli are small accumulations of fibroblasts and round cells (Fig. 1) which occasionally slightly invade the adjacent labyrinthine tissue. Nowhere have we seen any distortion or alteration in the usual architecture in either the cortex or the medulla. The tubular epithelium shows only slight degenerative changes with no evidences of tubular necrosis or regeneration.

Practically the same microscopic picture is found in Guinea Pigs 1, 2, 3, and 4 of this series (Table I), irrespective of the difference in size and number of doses given or the length of survival of the animal. The changes in Guinea Pig 2 are relatively more marked than in the others, although they are essentially of the same type.

TABLE I.

Arsenious Acid.

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.					Doses.	Days survived.	Termination.
	1	2	5	6	11			
1	5					1	3	Killed.
2	3	3		10		3	8	"
3	10		10		10	3	12	Died.
4	8		5			2	6	"

The proliferative changes in the kidney, therefore, after sublethal injections of arsenious acid are extremely slight and are confined to slight increase of nuclei in a somewhat shrunken glomerular tuft and to a slight accumulation of fibroblasts and round cells about the base of the glomeruli. There are no mitoses in the tubular epithelium, indicating a previous injury of this tissue with subsequent regeneration.

Arsenic Acid.

The gross and microscopic pictures of the kidneys of guinea pigs poisoned with lethal doses of arsenic acid are practically the same as those of arsenious acid. Vascular injury is the predominating change and there is but slight tubular necrosis. However, the study of the kidneys of guinea pigs that have received sublethal doses of arsenic

acid reveals characteristic changes that differentiate them rather sharply from those of arsenious acid. In the inner half of the cortex and in the boundary zone there is a considerable amount of young fibroblastic (connective) tissue extending for the most part along the medullary rays, but also invading the labyrinth to an appreciable degree. Some of the straight and convoluted tubules in these areas are compressed, but many more are dilated and may contain a granular precipitate (Fig. 2). The epithelium of some of these tubules shows degeneration and is flattened and cuboidal in type. In others it is basophilic and evidently of recent formation. Occasionally mitoses are seen. A few of the tubules in these areas appear as more or less solid cords of recently formed epithelial cells, which stain an intense blue with hematoxylin. The glomeruli in the kidneys of this series are practically normal.

TABLE II.

Arsenic Acid.

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.							Doses.	Days survived.	Termination.
	1	2	3	5	6	8	10			
2	2		4		4	10	10	5	13	Killed.
3	8							1	6	"
6	10	10		10				3	7	"

In Guinea Pig 2 (Table II), which received five doses of arsenic acid and survived 13 days, many of the blood vessels in the boundary zone are somewhat thickened. In certain glomeruli some of the capillary walls are apparently thickened also, but this is such an irregular finding and one that may be so variously interpreted that we hesitate to lay much emphasis upon it. The fibroblastic proliferation in the inner cortex and boundary zone is quite characteristic in this animal (Fig. 2). The amount of connective tissue proliferation in Guinea Pig 3 is less than in Guinea Pig 2, but the location and arrangement is the same. In Guinea Pig 6, on the other hand, there is slightly more connective tissue than in Guinea Pig 2, which may be due to the fact that Guinea Pig 6 survived the administration of three comparatively large doses of arsenic acid for 7 days.

In contrasting arsenious and arsenic acid, which resemble each other so closely in the character of the acute renal lesion, it appears that there are distinct differences in their action which are revealed in the process of repair. With arsenious acid, proliferative changes are almost wanting and are practically exclusively confined to the slight increase in nuclei of the glomerular tuft and to slight fibroblastic accumulation about the base of the glomeruli. With arsenic acid, on the other hand, proliferative activity of fibroblasts is appreciably more marked, and is associated with distortion of the tubules especially in the inner half of the cortex and boundary zone and with distinct (although slight) regeneration of tubular epithelium.

Sodium Cacodylate.

TABLE III.

Sodium Cacodylate.

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.				Doses.	Days survived.	Termination.
	1	2	5	6			
2	50	100		1,000	3	7	Died.
3	400		1,000		2	6	"
4	700				1	2	Killed.
5	700		700		2	6	Died.
6	500	500			2	3	"

The kidneys of guinea pigs poisoned with lethal doses of sodium cacodylate belong to the red type and resemble those of arsenious and arsenic acid. In the kidneys of guinea pigs which have survived 2 to 7 days, as is shown in Table III, we find practically the same slight proliferative activity that was observed in the animals which received sublethal doses of arsenious acid. There are occasional small accumulations of fibroblasts and round cells about the glomeruli, along the medullary rays, and in the boundary zone (Fig. 4). In some of the glomerular tufts there is a distinct increase in leukocytes of the endothelial type. There is nothing comparable to the fibroblastic proliferation with consequent distortion of tubules that was comparatively conspicuous in the animals poisoned with arsenic acid. However, in addition to the accumulation of fibroblasts there is more

necrosis of the tubular epithelium than with either arsenious or arsenic acid, and mitoses may be found in both the convoluted and the straight tubules.

There is but little difference, histologically, in the kidneys of the various guinea pigs of this series (Table III). In Guinea Pig 2 there are perhaps more and slightly larger accumulations of fibroblasts. Mitoses are found in the tubular epithelium of all five animals, but they are more numerous in Guinea Pig 6.

There is scarcely any fibroblastic proliferation, therefore, in the animals poisoned with sublethal doses of sodium cacodylate as well as with arsenious acid,—in this respect differing from those poisoned with arsenic acid. But, in addition, there is distinct reparative activity on the part of the tubular epithelium, indicating that this tissue has been injured by the drug, and in this respect differing from guinea pigs poisoned with sublethal doses of both arsenious and arsenic acids, in which the tubular epithelium is but little affected.

Salvarsan.

We have but one instance in which a sublethal dose of salvarsan was given and the animal allowed to live for more than 24 hours. This guinea pig received 150 mg. of the drug per kilo of body weight and was killed 48 hours after its administration.

The acute salvarsan kidney is a red kidney. In this one example of an early stage in the process of repair the glomeruli are large and rather irregular. The tufts on the whole are not shrunken and there is an accumulation of endothelial leukocytes in some of them. Many of the walls of the tuft capillaries are hyaline and distinctly thickened. About the base of some of the glomeruli there is a collection of fibroblasts and round cells which in some instances extends slightly outward into the labyrinth between the convoluted tubules. In the boundary zone and along the inner portion of the medullary rays are small irregular patches of young fibroblasts (Fig. 5), but there is no distortion of the tubular structures. The tubular epithelium is markedly degenerated but there is no definite epithelial necrosis, and since we have seen no mitoses we may infer that the tubular epithelium was not seriously injured. It should be stated in this

connection, however, that this animal received the dose of salvarsan intraperitoneally and that the compound was absorbed relatively slowly, for when the animal was killed 2 days after injection, the abdominal cavity contained a quantity of unabsorbed drug.

The evidences of repair, seen in the kidneys of guinea pigs after salvarsan injection, are therefore chiefly the changes in the glomeruli and the irregular proliferation of fibroblasts about the glomeruli and in the boundary zone.

Neosalvarsan.

The acute neosalvarsan kidney resembles those of arsenious acid and salvarsan. In Guinea Pig 1, killed on the 2nd day of survival, as shown in Table IV, we find definite signs of a proliferative activity

TABLE IV.

Neosalvarsan.

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.		Doses.	Days survived.	Termination.
	1	5			
1	300		1	2	Killed.
2	190	500	2	6	Died.

in the kidney in addition to the acute lesion which is still present. About the base of the glomeruli are small accumulations of fibroblasts and round cells. In addition there is a slight diffuse infiltration of the same character along the medullary rays in both cortex and medulla which in some areas invades the adjacent labyrinthine tissue. The glomeruli themselves are extremely irregular; some are of normal size, while others are markedly shrunken with a contracted tuft filling approximately three-quarters of the capsular space. The tubular epithelium is considerably degenerated and in the inner portion of the cortex there is some individual cell disintegration of the epithelium of the loops of Henle and a slight degree of necrosis. A few mitotic figures are seen.

The changes in Guinea Pig 2 are of the same general character as those in Guinea Pig 1, although of a lesser degree. This may be

due to the much smaller initial dose. The capsules of Bowman and the capillary walls of some of the glomerular tufts are slightly hyaline and thickened. There are small, irregular patches of fibroblasts and round cells about the glomeruli, along the medullary rays, and in the boundary zone. The tubular epithelium is degenerated with occasional slight necrosis. No mitoses are seen.

The changes in the process of repair after neosalvarsan resemble in general those after salvarsan, differing only in the greater irregularity of the glomeruli and the somewhat greater degree of tubular necrosis which is most marked in the inner portion of the cortex. After both salvarsan and neosalvarsan there is a patchy irregular proliferation of fibroblasts in the boundary zone and cortex, but there is no distortion of the tubules as with arsenic acid.

Arsacetin.

TABLE V.

Arsacetin.

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.				Doses.	Days survived.	Termination.
	1	5	13	16			
1	125	250			2	6	Killed.
2	100	200	200	200	4	19	"
3	300				1	2	"

The kidneys of dogs and guinea pigs which have received lethal doses of arsacetin are pale and show a most extensive tubular necrosis with a subordinate vascular injury. In the reaction from a non-fatal dose of this compound there is an exceedingly active and prompt regeneration of the epithelium, especially in the loops of Henle, and to a somewhat less extent in the convoluted tubules and the tubules of the medulla (Figs. 6, 7, and 8). The glomerular capillaries are somewhat dilated and there may be a slight and irregular accumulation of fibroblasts and polyblasts about the base of the glomeruli, along the medullary rays, and in the boundary zone. In Guinea Pig 3 (Table V), killed on the 2nd day, there is already marked activity of the tubular epithelium throughout the cortex and to a less extent in

the medulla. There is practically no vascular alteration and no fibroblastic proliferation in this animal. Guinea Pig 1 was killed on the 6th day after having received two doses of arsacetin. The kidney sections of this animal show a moderate regeneration of the tubular epithelium, almost exclusively confined to the loops of Henle and the medullary tubules. About the base of some of the glomeruli there is a very slight degree of fibroblastic proliferation. Four moderate sized doses of arsacetin were given to Guinea Pig 2 in 16 days, and the animal was killed on the 19th day (Table V). In this animal the regenerative activity of the tubular epithelium has reached a remarkable degree. Mitotic figures are found in practically every microscopic field (4 mm. Zeiss objective; No. 4 ocular) and they are particularly numerous in the straight tubules of the cortex and medulla (Figs. 6, 7, and 8). Many of the tubules along the medullary rays show entirely new epithelium and some of these tubules are almost solid masses of new cells. Degeneration with some necrosis and desquamation of cells is still present in some of the convoluted tubules and limbs of Henle and there are many hyaline casts in the boundary zone and medulla. In the boundary zone, along the medullary rays, and about the base of some of the glomeruli there is a rather diffuse proliferation of fibroblasts and infiltration of polyblasts which irregularly invade the labyrinth in some areas. Several of the renal tubules of this animal contain the parasite which we have referred to and which must be taken into consideration in interpreting the proliferative changes about the glomeruli.

Atoyxl.

The acute lesion in the kidneys of dogs and guinea pigs poisoned with lethal amounts of atoxyl is predominantly tubular, but the vascular changes are by no means inconspicuous. In guinea pigs which have received sublethal doses of atoxyl, there is a rapid attempt at regeneration of the tubular epithelium which is shown in both animals of this series (Table VI). Many of the straight tubules are dilated and lined with low cuboidal basophilic staining cells (Fig. 9), and there are numerous mitoses in both cortical and medullary epithelium (Figs. 9, 10, and 11). There is a well marked leukocytic exudate

in the interstitial tissue as well as in some of the tubules of Guinea Pig 3 (Fig. 11); it is less prominent in Guinea Pig 1. In addition to the marked epithelial necrosis with regeneration and the cellular exudate caused by the injection of atoxyl, there is a definite fibroblastic proliferation along the medullary rays, in the boundary zone, and in the upper medulla (Figs. 11 and 12).

The territorial distribution of fibrosblastic proliferation in Guinea Pig 3 corresponds with that in Guinea Pig 1. The process, however, is distinctly more pronounced, as might be expected, the animal having received three doses of atoxyl in 8 days. The fibroblasts are somewhat diffusely distributed throughout the lower edge of the cortex, and along the boundary zone and upper medulla. Some of the tubules in the area of fibroblastic proliferation are compressed,

TABLE VI.

Atoxyl.

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.			Doses.	Days survived.	Termination.
	1	2	5			
1	100			1	3	Killed.
3	50	50	50	3	8	"

while others are slightly dilated (Figs. 9 and 12). The glomeruli are irregular in both animals. Some are quite large, others are shrunken. Many of the tuft capillaries are dilated and the walls moderately thickened. About the base of the glomeruli are slight fibroblastic accumulations which in a few areas invade the adjacent labyrinth.

The process of repair in the kidneys of guinea pigs poisoned with sublethal doses of atoxyl resembles that of arsacetin in the prompt and marked regeneration of tubular epithelium. Moreover, there is a definite proliferation of fibroblasts which recalls the changes caused by arsenic acid, and in addition there is a well marked exudation of polymorphonuclear leukocytes into the interstitial tissue and tubules.

Arsenophenylglycine.

Arsenophenylglycine acts somewhat differently in guinea pigs and in dogs. With lethal amounts of the drug the kidneys of dogs and guinea pigs are both pale, with a predominant tubular necrosis and a relatively subordinate vascular injury. In the guinea pig, however, after a comparable sublethal dose of the compound there is but little tubular necrosis and but slight vascular injury. Consequently, after such a dose of arsenophenylglycine the process of repair in the kidney consists only in recovery from a parenchymatous and fatty degeneration of the tubular epithelium with now and then a mitotic figure.

TABLE VII.

Arsenophenylglycine.

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.		Doses.	Days survived.	Termination.
	1	5			
1	250		1	2	Killed.
2	250		1	5	"
3	100	50	2	6	Died.
4	400		1	3	Killed.
5	300	300	2	6	Died.
6	500		1	4	Killed.

Such a course of events is followed after a single or a repeated sublethal dose (Table VII). In Guinea Pig 1, for instance, killed on the 2nd day, degeneration of tubular epithelium is marked, but there is practically no cell necrosis, and no mitotic figures are seen. Guinea Pig 2 received the same amount of arsenophenylglycine as Guinea Pig 1, but was not killed until the 5th day. The epithelial changes in the two animals are almost identical. In Guinea Pig 2 there is an increase of tuft nuclei, mostly of the polymorphonuclear variety, and about the base of some of the glomeruli are slight accumulations of fibroblasts. Guinea Pig 3 received two small doses of the compound, and the alterations here are much the same as in the first two animals. The leukocytic cells infiltrating the glomerular

tuft are mostly eosinophilic; there is a very irregular thickening of the wall of the glomerular capillaries and an extremely irregular and slight distribution of round cells and fibroblasts about the base of some of the glomeruli, along the straight vessels, and in the labyrinth. The tubular epithelium is swollen and granular and there is some necrosis of a disintegrative character in the epithelium of the outer cortex. An occasional mitotic figure is seen.

The acute changes in the kidneys of guinea pigs after sublethal doses of arsenophenylglycine consist almost entirely of parenchymatous and fatty degeneration of the tubular epithelium with but little cell necrosis, differing in this respect from a similar sublethal injury in the kidneys of dogs. The processes of repair after such an injury are, therefore, comparatively simple and do not involve any appreciable degree of cell regeneration. There is only a slight and very irregular interstitial fibroblastic proliferation.

DISCUSSION.

A study of the processes of repair in the kidneys of guinea pigs poisoned with sublethal doses of certain arsenical compounds furnishes additional information as to the character and location of the acute injury. The idea, which we have previously suggested, namely, that all arsenicals do not produce a purely vascular type of renal injury, is further substantiated by this series of experiments in which the regeneration of tubular epithelium plays a relatively conspicuous part. The participation of the epithelial tissue, however, is by no means the dominant feature in the recovery after sublethal doses of all arsenical compounds, but only after certain particular ones. In others, the acute injury is mainly vascular, and the reaction of the epithelial structures is but slight. Further, the interstitial proliferation of fibroblasts which occurs in a marked degree after injections of various arsenicals may be especially pronounced in the repair following a drug which, in lethal doses, causes but little vascular disturbance, as, for instance, arsacetin. Here, the initial and dominant injury is epithelial; there is only slight demonstrable alteration of the vascular structures. In the process of recovery, however, after the administration of sublethal doses of arsacetin, there may be quite

a marked proliferation of young fibroblasts diffusely distributed (Table V, Guinea Pig 2, and Fig. 8). This is also seen in the repair of the kidney following injections of atoxyl, although here the picture is more complicated, for atoxyl affects the vascular as well as the epithelial tissue of the kidney. The distribution of the proliferating fibroblasts after a more purely vascular injury such as that produced by arsenious or arsenic acid is confined more sharply to the boundary zone, with radiations along the medullary rays to the glomeruli. On the other hand, after arsacetin, the fibroblastic proliferation is more diffusely distributed throughout the cortex, although it may be more numerous in the boundary zone and along the medullary rays.

Taking all these observations into consideration, therefore, it would seem that a toxic agent like arsenic, may, in one or another of its various combinations, injure the vascular, epithelial, or interstitial (connective) tissue of the kidney, if we may judge of such an initial injury by the subsequent processes of repair. The relative distribution and extent of the initial injury may be difficult to determine until one studies various stages of the recovery of the kidney, in which the injured tissues are regenerating. This is particularly true in the case of injury of the connective tissue of the kidney.

After a sublethal dose of arsenious acid which produces an almost pure type of vascular injury in the kidneys, the return to normal is very rapid and there is only a slight fibroblastic proliferation about the base of the glomeruli to indicate a previous injury. Injections of sublethal amounts of salvarsan also cause a proliferation of fibroblasts of a more interstitial and rather patchy character, somewhat greater in extent and amount than with arsenious acid. On the other hand, with arsenic acid, there is a relatively large amount of fibroblastic proliferation, especially in the boundary zone, resulting in the compression or dilatation of some of the tubules, and in addition a slight although definite regeneration of tubular epithelium. Sodium cacodylate in sublethal amounts causes essentially the same slight fibroblastic changes seen after arsenious acid, but a well marked regeneration of tubular epithelium as well. With all these four compounds, arsenious and arsenic acid, salvarsan, and sodium cacodylate, there are slight but fairly regular glomerular changes, consisting of

a slightly swollen tuft and an increase in the tuft nuclei. With neosalvarsan, however, the glomeruli are extremely irregular, some being very large, others contracted and shrunken. There is a slight interstitial fibroblastic proliferation, comparable to that of salvarsan and a slight regeneration of tubular epithelium. After sublethal injections of atoxyl, there is very marked regenerative activity of the tubular epithelium, a leukocytic exudate, and a definite and rather diffuse interstitial proliferation of fibroblasts with dilatation or compression of adjacent tubules. Following a sublethal injection of arsacetin, there is a conspicuous and prompt regeneration of tubular epithelium and a relatively diffuse interstitial proliferation of fibroblasts. Unfortunately, we must omit the experiments of arsenophenylglycine on the guinea pig in this consideration, for the reasons stated above.

It would seem, therefore, that arsenicals which produce an injury that is primarily vascular may lead to only a slight subsequent proliferation of tissue (arsenious acid). However, other arsenicals which produce an acute injury that is vascular, may lead to distinct interstitial proliferation (arsenic acid, salvarsan, and neosalvarsan). Moreover, an arsenical that produces an acute injury that is primarily vascular may also produce injury of the tubular epithelium which in the stages of repair dominates the picture to the exclusion of any extensive interstitial proliferation (sodium cacodylate).

On the other hand, arsenicals that produce primarily an injury of tubular epithelium cause a marked subsequent regeneration of this tissue, and may cause in addition an interstitial fibroblastic proliferation (arsacetin). But an arsenical compound that produces a marked vascular injury in addition to the picture of tubular necrosis may lead to a relatively marked interstitial proliferation as well as the extensive epithelial regeneration (atoxyl).

In connection with the fibroblastic proliferation described in the processes of repair in these kidneys, we must refer to the presence of a parasite in the kidneys of some guinea pigs.[2] This parasite is only occasionally found in guinea pigs, but we believe that it may cause irregular accumulations of fibroblasts and round cells about the base of some of the glomeruli and in the neighboring labyrinthine tissue. These accumulations resemble those seen after sublethal injection

of arsenious acid, but they are much more irregular. Therefore, we wish to be particularly cautious in a final interpretation of our results and to take into consideration the possibility that some of the fibroblastic proliferation we have seen in the kidneys of guinea pigs after sublethal injections of these arsenicals may be due in part to this renal parasite.

We have shown that after acute lethal injury with various arsenicals, two types of kidneys could be distinguished, both grossly and histologically, namely, the red and the pale. Further, in the reaction after a sublethal injury inflicted with these compounds these two main types of kidney alteration may still be differentiated by the processes of repair. The administration of those compounds which produce a predominantly pale kidney is followed by a most prompt and pronounced regeneration of tubular epithelium with a varying degree of diffuse fibroblastic proliferation. The compounds that produce a predominantly red kidney are followed by proliferation of fibroblasts with but slight regeneration of the tubular epithelium. The distribution of the fibroblasts in the reparative stages apparently corresponds in some degree to the initial injury. After a more or less predominant vascular injury the fibroblasts are usually found about the glomeruli, along the medullary rays, and in the boundary zone. With other compounds that show little evidence of an acute vascular injury, the connective tissue injury may be more pronounced, and in recovery from such an injury the fibroblasts are diffusely distributed throughout the cortex and medulla in a typical interstitial manner. But, just as there are innumerable combinations of vascular and tubular and probably connective tissue injury in the acute arsenic kidneys, so there are innumerable combinations of the different tissue elements in the processes of repair. Hence, we suggest that the character and distribution of renal injury produced by arsenical compounds as indicated by the processes of repair are bound up in the chemical constitution of these compounds. Further, that each particular compound as far as the kidney is concerned, acts as a more or less specific toxic agent, as shown by the character and distribution of the renal lesions.

SUMMARY.

1. The processes of repair in the kidneys of guinea pigs after sublethal doses of certain arsenical compounds indicate that all arsenicals do not produce a purely vascular type of renal injury.

2. While some arsenicals produce a predominantly vascular injury and others produce a predominantly tubular injury, both these tissue elements are undoubtedly always affected, although in varying proportion. In addition, the interstitial connective tissue is probably always affected. The diffuse proliferation of this tissue may be relatively conspicuous in the processes of repair after arsenicals that cause but slight vascular injury.

3. All red kidneys do not necessarily show identical pictures during the processes of repair; the same is true of pale kidneys.

4. The mode of action of an arsenical compound as a renal toxic agent is bound up with the chemical constitution of the compound.

EXPLANATION OF PLATES.

The illustrations are all from untouched microphotographs.

Plate 71.

Fig. 1. Arsenious acid. Guinea Pig 2. Section from the outer cortex. There is a regular and definite although slight accumulation of round cells and fibroblasts about the glomeruli. The nuclei of the glomerular tuft show a slight increase. The tubular epithelium is normal. × 210.

Fig. 2. Arsenic acid. Guinea Pig 2. Section from the inner cortex. There is a slight interstitial proliferation of connective tissue, and the renal tubules in this area are somewhat distorted, some being dilated and others compressed. The tubular epithelium and glomeruli are practically normal. × 210.

Plate 72.

Fig. 3. Arsenic acid. Guinea Pig 6. Section from the boundary zone. There is a small but conspicuous proliferation of connective tissue with consequent dilatation and compression of the renal tubules in this area. The renal epithelium is swollen and shows parenchymatous degeneration. × 210.

Fig. 4. Sodium cacodylate. Guinea Pig 2. Section from the outer cortex. The glomerular tuft is slightly swollen and shows a slight increase of tuft nuclei. There is a definite and regular although slight infiltration of round cells and fibroblasts around the base of the glomeruli. The renal epithelium is slightly swollen and degenerated. × 210.

PLATE 73.

FIG. 5. Salvarsan. Guinea Pig 1. Section from the boundary zone. There is an irregular patchy proliferation of connective tissue. The renal epithelium is practically normal. × 210.

FIG. 6. Arsacetin. Guinea Pig 2. Section from the outer cortex. There is a marked degeneration and regeneration of the tubular epithelium with numerous mitotic figures. There is a granular precipitate in the lumen of the tubules which appear slightly dilated. The glomeruli are normal in appearance. There is a slight diffuse proliferation of fibroblasts. × 210.

PLATE 74.

FIG. 7. Arsacetin. Guinea Pig 3. Section from the midcortex. There is a moderate degeneration and marked regeneration of the tubular epithelium with numerous mitotic figures. There is a granular precipitate in the lumen of the tubules. × 210.

FIG. 8. Arsacetin. Guinea Pig 2. Section from the upper medulla. There is marked regeneration of the tubular epithelium with numerous mitotic figures. There is a slight but definite interstitial proliferation of fibroblasts. × 210.

PLATE 75.

FIG. 9. Atoxyl. Guinea Pig 3. Section from the inner cortex. There is a conspicuous dilatation of some of the tubules with a granular precipitate in the lumen. The tubular epithelium shows regeneration with numerous mitotic figures. The glomeruli appear normal. × 210.

FIG. 10. Atoxyl. Guinea Pig 1. Section from the outer cortex. There are two adjacent mitotic figures in the epithelium of a convoluted tubule. × 1,470.

PLATE 76.

FIG. 11. Atoxyl. Guinea Pig 3. Section from the boundary zone. There is a conspicuous leukocytic infiltration in the tubules and interstitial tissue. The tubular epithelium shows numerous mitotic figures. There is some interstitial proliferation of the connective tissue. × 378

FIG. 12. Atoxyl. Guinea Pig 3. Section from the boundary zone. There is a diffuse interstitial proliferation of connective tissue. The tubular epithelium shows several mitotic figures. × 378.

EXPERIMENTS WITH POLIOMYELITIS IN THE RABBIT.

By M. J. ROSENAU, M.D., and L. C. HAVENS.

(*From the Department of Preventive Medicine and Hygiene, Harvard Medical School, Boston.*)

Plates 77 to 80.

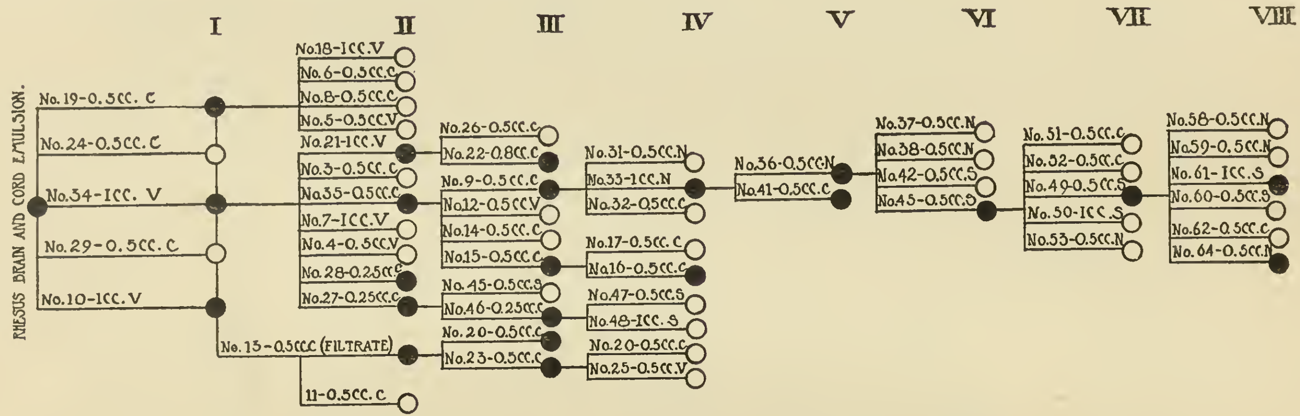
(Received for publication, January 13, 1916.)

Inoculation of the common laboratory animals with the virus of poliomyelitis has met with so little success that the disease has been generally regarded as exclusively limited to man and the monkey. Attempts have been made to transmit the virus to many animals, but of all the animals tested, positive results have thus far been recorded only for monkeys, rabbits, and, perhaps, guinea pigs.

Krause and Meinicke[1] reported the passage of a strain of virus obtained from a human case through seven generations in rabbits. Lentz and Huntemüller[2] report having successfully transferred the virus from rabbit to rabbit by various methods of inoculation. They found the alterations in the brain and spinal cord to be slight as compared with those in monkeys. On the other hand, Römer and Joseph,[3] Landsteiner and Levaditi,[4] Leiner and von Wiesner,[5] and Flexner and Lewis[6] all failed to transfer the disease to rabbits. The most striking results, perhaps, have been reported by Marks,[7] who carried a strain of poliomyelitic virus derived from a *rhesus* monkey through seven generations of young rabbits varying in weight from 350 to 550 gm. The animals that succumbed developed no paralysis, but died in convulsions. No lesions definitely characteristic of poliomyelitis could be found on microscopic examination. Marks says: "The disease thus produced in rabbits cannot be recognized as poliomyelitis," although he concludes that filtrates of the nervous tissues of monkeys dying from experi-

---

[1] Krause, P., and Meinicke, E., *Deutsch. med. Wchnschr.*, 1909, xxxv, 1825.
[2] Lentz and Huntemüller, *Ztschr. f. Hyg. u. Infectionskrankh.*, 1910, lxvi, 481.
[3] Römer, P. H., and Joseph, K., *München. med. Wchnschr.*, 1910, lvii, 2685.
[4] Landsteiner, K., and Levaditi, C., *Compt. rend. Soc. de biol.*, 1909, lxvii, 787.
[5] Leiner, C., and von Wiesner, R., *Wien. klin. Wchnschr.*, 1909, xxii, 1698.
[6] Flexner, S., and Lewis, P. A., *Jour. Exper. Med.*, 1910, xii, 227.
[7] Marks, H. K., *Jour. Exper. Med.*, 1911, xiv, 116.



TEXT-FIG. 1. Chart showing the progress of the virus through eight generations of rabbits. C, intracerebral inoculation; V, intravenous inoculation; N, nasal insufflation; S, injection into sciatic nerve sheath.

mental poliomyelitis are not wholly innocuous to young rabbits. Marks further states that "not all strains of the virus can be transmitted successfully to even a small fraction of individuals of all varieties of domesticated rabbits." This fact, which we have corroborated, may explain the negative results of other investigators.

Römer and Joseph[3] have observed that guinea pigs in the laboratory occasionally die of a paralytic disease. They were unable to transfer poliomyelitic virus from monkeys to guinea pigs, although Römer[8] found that the spontaneous paralysis was due to a filterable virus. Neustaedter[9] claims to have carried a strain from a guinea pig presumably infected through contact with a monkey into one other guinea pig and back again to a monkey.

In the course of our experiments we have inoculated a few guinea pigs. The lesions in those that succumbed were somewhat similar to the lesions seen in the rabbits. This part of our work is not sufficiently advanced to warrant conclusions. Moreover, we have no criterion by which to establish what is and what is not poliomyelitis, except by reproducing the disease in monkeys. It is, therefore, hazardous to affirm or deny the identity with poliomyelitis of these aberrant conditions in other animal species.

The difficulty of obtaining monkeys on account of the war led us to consider other animals that might be susceptible and therefore suitable for experimental purposes. We selected the rabbit because successful results had previously been obtained with these rodents and because rabbits offered a good chance to compare the action of poliomyelitic with rabic virus. We accordingly inoculated rabbits with poliomyelitic virus from a *rhesus* monkey infected by intracranial inoculation with a strain sent us from The Rockefeller Institute for Medical Research by Dr. Harold L. Amoss. We have obtained positive results in young rabbits and have succeeded in transferring the virus from rabbit to rabbit through eight successive generations.

Intracranial inoculations have been used for the most part, although infection has taken place through intravenous injection, also through injection into the sciatic nerve sheath, and even after introduction of the virus into the anterior nares, upon the uninjured nasal mucosa. A few intraperitoneal injections were tried, but with negative results.

[8] Römer, P. H., *Ergebn. d. inn. Med. u. Kinderh.*, 1912, viii, 1.

[9] Neustaedter, M., *Jour. Am. Med. Assn.*, 1913, lx, 982.

All inoculations are by no means successful. Over one-half fail; we obtained positive results in twenty-two rabbits out of a total of fifty-four inoculated in various ways. Of five rabbits inoculated with the virus from the *rhesus* monkey two have failed to show symptoms, although kept under observation for 5 months.

The age incidence of poliomyelitis in man is indicated by the name "infantile paralysis." In our experimental work we obtained positive results in young rabbits only. We failed to infect three full grown rabbits with *rhesus* virus, although this same virus caught in three out of five young rabbits. Furthermore, rabbit virus from the second and third generations was transferred to rabbits 8 weeks old with negative results. Thereafter we used only young animals under 6 weeks old in our experiments. There seems to be a parallel between rabbits and man as far as susceptibility of the young is concerned. These facts seem to furnish a striking example of natural immunity acquired during the period of adolescence.

The incubation period in our observations of twenty-two rabbits has been variable, the shortest being 2 days, the longest, 41. In this respect our experience is similar to that of Lentz and Huntemüller,[2] who found the incubation period to be of uncertain length, sometimes as long as 2 months, but usually between 7 and 11 days. The average period of incubation in our observations was 12 days. Curiously enough, the shortest period, 2 days, as well as the longest, 41 days, was after intracranial injections.

The virus so far has given no evidence of increasing adaptation to rabbits, or of becoming fixed to any degree, the number of failures being as great, and the period of incubation as variable, in the eighth generation as in the first.

The symptoms also vary. They can, however, be divided roughly into two classes: (1) A type which we designate the progressive type, in which the rabbit first becomes inactive, loses weight, and appears weak. This is followed by partial or complete paralysis of one or more of the extremities (Fig. 1), which usually progresses until death. This corresponds somewhat to the syndrome most commonly seen in experimental poliomyelitis in monkeys. The duration of the disease varies from 1 or 2 days to 1 week. The paralysis is usually flaccid, but occasionally it is spastic. Sometimes the paralysis be-

gins locally. As a rule, it is easy to determine that the paralysis is a true palsy and not simply "weakness." (2) The other group of symptoms, which we designate the fulminating type, is more explosive in character, develops suddenly, and terminates in a very short time; it never extends over more than 2 days and usually lasts only a few hours. The overshadowing symptoms are extreme weakness and marked dyspnea. As an illustration of the rapid course of the symptoms of the fulminating type the case of Rabbit 21 is cited. This rabbit was inoculated intravenously on Aug. 18 with 1 cc. of a rich emulsion of virus from a first generation rabbit (No. 34). On Sept. 12, 25 days later, it still appeared to be normal. In about an hour after this observation it was found by one of us lying flat on its side, breathing in slow, labored gasps. There was great general weakness, the rabbit being unable to raise its head, but no paralysis of the extremities was demonstrable. Within a few minutes the animal was dead, the whole syndrome lasting less than an hour. This is an extreme instance of the fulminating type, and is of special interest since it follows a long period of incubation. We have seen combinations of the two types with symptoms resembling Landry's paralysis.

Most of the animals in which the symptoms lasted over 2 or 3 days lost weight rapidly. In some cases the loss of weight was the first observed symptom.

That the rabbit virus is filterable is shown by the protocol of Rabbit 13 of the second generation. This rabbit was inoculated intracranially with 0.5 cc. of a Berkefeld filtrate of brain and cord emulsion from Rabbit 10. Six days later the hind legs became paralyzed, and the animal died 2 days after onset with symptoms of the fulminating type. The unfiltered virus from Rabbit 13 proved infectious for two other rabbits (Nos. 20 and 23).

We have inoculated a monkey with virus from Rabbit 49 of the seventh generation. The monkey died after an incubation period of 4 weeks, with symptoms somewhat resembling the fulminating type seen in the rabbits. The gross pathological findings were congestion of the pia, especially in the region of the medulla, and hyperemia and hemorrhage of the gray matter of the medulla and cord. The microscopic lesions were similar to those seen in the rabbits and not typi-

cal of experimental poliomyelitis in monkeys. The monkey was a South American species which we have since found to be highly resistant to poliomyelitic virus derived from *rhesus* monkeys. This interesting observation is being made the subject of further study.

Flexner found that *Capucinus* monkeys were resistant, although *Cebus*, another South American species, were susceptible, but less so than Old World monkeys. He therefore concludes that the *Platyrrhines* are less susceptible than the *Catarrhines*.[10]

The intracranial inoculations were done by making a small incision in the skin, near the midline, drawing this to one side, and introducing the needle of the syringe directly through the frontal bone, which is thin and soft in young rabbits. The virus is then injected slowly into the region of the lateral ventricle. On withdrawing the needle, the skin slides back and acts as a valve to cover the small opening in the bone.

The emulsion of virus in all cases was made by grinding in a mortar, portions of the brain and cord with salt solution, and filtering through several layers of sterile gauze.

In Table I are briefly summarized the results of the rabbits which succumbed to intracranial inoculation.

Fourteen rabbits inoculated intracranially failed to show symptoms.

The intravenous inoculations were made into the posterior auricular vein with an emulsion of the central nervous matter prepared as above described.

Table II contains a condensed summary of the rabbits which succumbed to intravenous inoculations.

Six rabbits inoculated intravenously failed to show symptoms.

In order to inject the virus into the sciatic nerve sheath, a short incision was made in the skin above the nerve, the tissues were dissected sufficiently to render the nerve visible, and the needle was then inserted into the sheath in the central direction. The emulsion was prepared as above

Table III gives a condensed summary of the rabbits which succumbed as a result of injection into the sciatic nerve sheath.

Five rabbits inoculated into the sciatic nerve sheath failed to develop symptoms.

[10] Flexner, S., *Jour. Am. Med. Assn.*, 1910, lv, 1105.

TABLE I.

Intracranial Inoculation.

No. of rabbit.	Amount of emulsion.	Rabbit generation.	Period of incubation.	Type of symptoms.*
	cc.		*days*	
19	0.5	I	19	P.
35	0.5	II	5	P.
27	0.25	II	31	F.
28	0.25	II	34	F.
13	0.5	II	6	F.
20	0.5	III	41	F.
23	0.5	III	9	P.
22	0.8	III	10	P.
46	0.25	III	10	Convulsions.
15	0.5	III	2	F.
9	0.5	III	14	P.
16	0.5	IV	13	P.
41	0.5	V	12	P.

* In this and the following tables, the letter P indicates symptoms of the progressive type; F, symptoms of the fulminating type.

TABLE II.

Intravenous Inoculation.

No. of rabbit.	Amount of emulsion.	Rabbit generation.	Period of incubation.	Type of symptoms.
	cc.		*days*	
34	1.0	I	18	F.
10	1.0	I	3	F.
21	1.0	II	25	F.

TABLE III.

Inoculation into the Sciatic Nerve Sheath.

No. of rabbit.	Amount of emulsion.	Rabbit generation.	Period of incubation.	Type of symptoms.
	cc.		*days*	
43	0.5	VI	8	P.
49	0.5	VII	6	F.
61	1.0	VIII	20	F.

In order to introduce the virus into the nose, the following procedure was adopted. The rabbit was lightly etherized, held on its back, and the emulsion of the virus then dropped into the anterior nares from an ordinary medicine dropper, care being taken not to injure the mucosa. There was usually a little sneezing immediately after the fluid was introduced.

Table IV is a condensed summary of the three rabbits which succumbed as a result of intranasal insufflation.

TABLE IV.

Intranasal Insufflation.

No. of rabbit.	Amount of emulsion.	Rabbit generation.	Period of incubation.	Type of symptoms.
	cc.		*days*	
33	0.5	IV	2	F.
36	0.5	V	2	F.
64	0.25	VIII	2	F.

Six rabbits inoculated by intranasal insufflation failed to develop symptoms.

It will be seen that, judging from the limited data at hand, this is a particularly virulent way of infecting rabbits with the virus. The period of incubation is short—only 2 days—and the symptoms in all three cases were of the fulminating type. These represent only three out of nine rabbits tested by introducing the virus into the nose. The other six failed to take. Particular attention was paid to the lungs of the rabbits in this group at autopsy. There was no evidence of pneumonia, or even of congestion of the lungs. On microscopical examination it was found that the medulla was markedly congested, whereas the cord was only slightly affected.

The gross lesions consist of injection of the vessels of the pia, hyperemia of the gray matter of the medulla and cord, and more or less marked edema throughout the brain and cord. The microscopic lesions are distributed rather uniformly throughout the gray matter of the cord and medulla in the progressive type. In the fulminating group (those exhibiting symptoms of respiratory failure without

paralysis of the skeletal muscles), the lesions are more marked in the medulla.

The microscopic lesions consist of capillary congestion, focal hemorrhages into the gray matter, degeneration of the large motor cells, and infiltration with cells of uncertain origin (Figs. 2, 3, 4, 5, 6, 7, and 8). These cells for the most part seem to be proliferated glia cells, and therefore appear to be different from the infiltrating lymphocytes of the lesions of poliomyelitis in man. The congestion of the capillaries and small arterioles is conspicuous. The infiltrating cells when stained with eosin and methylene blue have large, vesicular nuclei with a number of conspicuous chromatin granules. The cytoplasm is scanty and homogeneous. They are scattered throughout the gray matter, and are also grouped in satellite arrangement around the nerve cells, but the perivascular infiltration, so typical of the lesions of poliomyelitis in man and the monkey, is absent in the rabbit. Punctate hemorrhages are numerous, sometimes every vessel in a field being ruptured. The nerve cells show all stages of degeneration. Chromatolysis is common and satellitosis is an almost constant feature. A moderate degree of round cell infiltration is occasionally seen in the meninges. The accompanying plates illustrate the different features of the lesions. The complete picture gives the impression of a severe intoxication of the gray matter of the cord and medulla.

The following are the protocols in brief of the rabbits that succumbed, arranged chronologically, as in Text-fig. 1.

Rabbit 34.—Age 6 wks. Weight 660 gm. 1st generation. 1 cc. of *rhesus* virus intravenously. Incubation period 18 days. Died in the night with no observed symptoms.

Rabbit 19.—Age 5 wks. Weight 600 gm. 1st generation. 0.5 cc. of *rhesus* virus intracranially. Incubation period 19 days. Death in 4 days. Paralysis of front legs with respiratory symptoms.

Rabbit 10.—Age 4 wks. Weight 550 gm. 1st generation. 1 cc. of *rhesus* virus intravenously. Incubation period 3 days. Died in the night with no observed symptoms.

Rabbit 21.—Age 5 wks. Weight 600 gm. 2nd generation. 1 cc. of virus of Rabbit 34 intravenously. Incubation period 25 days. Death in 1 hour. Symptoms very explosive in character; complete prostration and marked dyspnea.

Rabbit 35.—Age 6 wks. Weight 730 gm. 2nd generation. 0.5 cc. of virus of Rabbit 34 intracranially. Incubation period 5 days. Hind legs became

paralyzed. Paralysis remained stationary for 5 days, then showed tendency to improvement. Chloroformed.

Rabbit 27.—Age 3 wks. Weight 290 gm. 2nd generation. 0.25 cc. of virus of Rabbit 34 intracranially. Incubation period 31 days. Death in 1 day. Paralysis and dyspnea.

Rabbit 28.—Age 3 wks. Weight 255 gm. 2nd generation. 0.25 cc. of virus of Rabbit 34 intracranially. Incubation period 34 days. Death in 1 day. Weakness and dyspnea.

Rabbit 13.—Age 4 wks. Weight 540 gm. 2nd generation. 0.5 cc. of Berkefeld filtrate of emulsion of virus of Rabbit 10 intracranially. Incubation period 6 days. Death in 2 days. Paralysis of hind legs and respiratory distress.

Rabbit 20.—Age 5 wks. Weight 645 gm. 3rd generation. 0.5 cc. of virus of Rabbit 13 intracranially. Incubation period 41 days. Death in 1 day. Explosive symptoms; prostration and marked dyspnea.

Rabbit 23.—Age 4 wks. Weight 410 gm. 3rd generation. 0.5 cc. of virus of Rabbit 13 intracranially. Incubation period 9 days. Death in 5 days. General weakness and dyspnea.

Rabbit 22.—Age 5 wks. Weight 620 gm. 3rd generation. 0.8 cc. of virus of Rabbit 21 intracranially. Incubation period 10 days. Death in 7 days. Paralysis progressive.

Rabbit 46.—Age 5 wks. Weight 380 gm. 3rd generation. 0.25 cc. of virus of Rabbit 27 intracranially. Incubation period 10 days. Death in 1 day. Died in convulsions.

Rabbit 15.—Age 6 wks. Weight 520 gm. 3rd generation. 0.5 cc. of virus of Rabbit 35 intracranially. Incubation period 2 days. Symptoms progressed rapidly; respiratory distress and great weakness. Chloroformed.

Rabbit 9.—Age 4 wks. Weight 440 gm. 3rd generation. 0.5 cc. of virus of Rabbit 35 intracranially. Incubation period 14 days. Death in 7 days. Progressive paralysis with respiratory distress.

Rabbit 16.—Age 4 wks. Weight 430 gm. 4th generation. 0.5 cc. of virus of Rabbit 15 intracranially. Incubation period 13 days. Death in 8 days. Symptoms progressive in type with dyspnea.

Rabbit 33.—Age 6 wks. Weight 620 gm. 4th generation. 0.5 cc. of emulsion of virus of Rabbit 9, half the amount dropped in each nostril. Incubation period 2 days. Death in 1 day. Symptoms of the fulminating type with paralysis of hind legs towards the last.

Rabbit 41.—Age 5 wks. Weight 560 gm. 5th generation. 0.5 cc. of emulsion of virus of Rabbit 33 intracranially. Incubation period 12 days. Death in 4 days. Symptoms of progressive type.

Rabbit 36.—Age 4 wks. Weight 380 gms. 5th generation. 0.25 cc. of heavy emulsion of virus of Rabbit 33 in each nostril. Incubation period 2 days. Death in 2 days. Symptoms of fulminating type with paralysis.

Rabbit 43.—Age 5 wks. Weight 450 gm. 6th generation. 0.5 cc. of emulsion of virus of Rabbit 36 injected into sciatic nerve sheath. Incubation period 8

days. Symptoms progressive in type. Complete paralysis on 3rd day. Chloroformed.

Rabbit 49.—Age 4 wks. Weight 310 gm. 7th generation. 0.5 cc. of emulsion of virus of Rabbit 43 into sciatic nerve sheath. Incubation period 6 days. Death in 2 days. Symptoms fulminating in type.

Rabbit 64.—Age 4 wks. Weight 400 gm. 8th generation. 0.25 cc. of emulsion of virus of Rabbit 49 into each nostril. Incubation period 2 days. Death in 2 days. Symptoms of fulminating type.

Rabbit 61.—Age 4 wks. Weight 340 gm. 8th generation. 1.0 cc. of emulsion of virus of Rabbit 49 into sciatic nerve sheath. Incubation period 20 days. Died in night with no observed symptoms.

DISCUSSION AND SUMMARY.

The poliomyelitic virus obtained from an experimental monkey has been passed through eight generations in rabbits. It shows no signs of dying out. On the other hand, it gives no evidence of becoming more pathogenic to the species through successive passage. The period of incubation remains variable and the percentage of takes has not increased. Whether eventually a virus can be obtained which is of heightened virulence to rabbits is problematic.

All inoculations are by no means successful. The animals show great individual differences in susceptibility to the virus, as is evidenced by the fact that out of fifty-four rabbits inoculated, only twenty-two, or about 40 per cent, succumbed. This fact may explain the negative results of other investigators. At several points in the series of experiments it was thought that the strain had died out. As many as six rabbits have been inoculated one after the other before the virus would catch again.

The age of the rabbits is important in considering the susceptibility. From the limited data at our command, adult rabbits are resistant, and there appears to be an abrupt increase in resistance between the age of 6 and 8 weeks; that is, rabbits under 6 weeks are more susceptible to the virus. There seems to be a parallel between the age incidence of this disease in rabbits and spontaneous poliomyelitis in man. The age incidence of poliomyelitis in man is indicated by the term "infantile paralysis."

Several methods of inoculation have proved successful; thus the rabbits have succumbed as a result of introducing the virus directly

into the brain, by injecting it into a peripheral nerve, or directly into the circulation, or by placing it upon the uninjured nasal mucosa.

The symptoms produced show more or less departure from the symptoms of poliomyelitis as seen in the spontaneous disease in man and in the experimental disease in the monkey. There are two distinct pictures recognizable. In one there is paralysis of one or more of the extremities which progresses until death, resembling somewhat the symptoms of the experimental disease in the monkey. This we have designated the progressive type. The other group is included in what we have called the fulminating type. The symptoms are explosive in character, with extreme weakness amounting to prostration, terminating in death in a few hours, attendant upon respiratory failure. The mode of inoculation seems to have little effect upon the type of symptoms produced.

The period of incubation is variable and apparently does not depend upon the method of inoculation. The period varied from 2 to 41 days, with an average of 12 days. The two extremes both occurred after intracranial injection. After intranasal insufflation the incubation period was short, being in each case 2 days, followed by symptoms of the fulminating type. The placing of the virus into the nose seems to be an effective method, but is as uncertain as other routes, as only three out of nine rabbits tested in this manner succumbed. The disease produced by this route was particularly virulent.

The virus shows no tendency to become fixed. The period of incubation is as variable in the eighth generation as in the first, and the virus has shown no tendency towards increasing virulence through successive passage, in these respects differing from the virus of rabies.

We have found the virus to be filterable. An emulsion of the central nervous matter of a rabbit of the first generation passed through a Berkefeld filter, and injected intracerebrally into another rabbit, resulted in death, preceded by symptoms of the fulminating type. Virus (unfiltered) from this rabbit was transferred successfully to two other rabbits.

The lesions, while definite and consistent throughout the series, lack the distinctive features of the pathologic picture of poliomyelitis in man and the monkey. Capillary congestion, punctate hemor-

rhages, degeneration of the motor cells, satellitosis, and more or less cellular infiltration of the gray matter of the cord and medulla are found, but perivascular infiltration is absent and the infiltrating cells are not lymphocytic in character.

One of the most striking features of this investigation is the way in which rabbits and monkeys react to the same virus. The disease in the rabbit presents certain clinical resemblances to the experimental disease in the monkey and also to the spontaneous disease in children. On the other hand, the symptoms show marked variation from those seen in the monkey and in man. The picture has not the same constancy in rabbits and could not in most cases be recognized clinically as poliomyelitis. There are still more marked differences in the pathology. While it is true that the brunt of the attack in the rabbit falls upon the gray matter of the cord and medulla, the appearance of the lesions under the microscope shows such differences from the lesions of experimental poliomyelitis in monkeys, as well as the natural disease in man, as to suggest two distinct infections. It is more reasonable, however, to assume that we are dealing with a modified form of poliomyelitis; that the rabbit reacts differently to the virus than the monkey or man; and that the disease produced in rabbits by us and others is in fact poliomyelitis. So far as we know, no other virus produces such differences in two animal species. Smallpox is so profoundly altered in the cow that it took almost 100 years to prove Jenner's assumption that cowpox is a modified form of smallpox. However, the pock of vaccinia is a correct counterpart both clinically and pathologically of the pock of variola. If the virus of poliomyelitis may be so altered in the rabbit as scarcely to be recognizable, may it not be still more profoundly changed in other animals? The conjecture then arises that poliomyelitis, instead of being limited naturally to man and experimentally to monkeys, may in fact occur in other animals in unnoticed or unrecognized form. If this should prove true, it may be a source of human infection and may help to solve the problem of prevention.

EXPLANATION OF PLATES.[11]

Plate 77.

Fig. 1. Rabbit 19. Paralysis of extremities.

Fig. 2. Rabbit 19. Medulla. Chromatolysis of the nerve cells. × 515.

Plate 78.

Fig. 3. Rabbit 9. Lumbar cord, showing capillary congestion and hemorrhage. × 180.

Fig. 4. Rabbit 9. Lumbar cord. Numerous punctate hemorrhages, and marked cell degeneration. × 385.

Plate 79.

Fig. 5. Rabbit 35. Lumbar cord. Cellular infiltration. × 180.

Fig. 6. Rabbit 9. Medulla. Satellitosis around degenerated nerve cells. × 515.

Plate 80.

Fig. 7. Rabbit 35. Satellitosis. × 515.

Fig. 8. Rabbit 19. Cervical cord. Degeneration of anterior horn cells with satellitosis. × 385.

---

[11] We are indebted to Dr. J. P. Bill for the microphotographic work.

FEEDING EXPERIMENTS WITH BACTERIUM PULLORUM. THE TOXICITY OF INFECTED EGGS.

By LEO F. RETTGER, Ph.D., THOMAS G. HULL, and WILLIAM S. STURGES.

(*From the Sheffield Laboratory of Bacteriology and Hygiene, Yale University, New Haven, and the Storrs Agricultural Experiment Station, Storrs.*)

(Received for publication, February 2, 1916.)

In a previous investigation on the bacteriology of the hen's egg[1] sufficient evidence was acquired to show that the contents of normal fresh eggs are, as a rule, sterile, and that even eggs which have been incubated artificially for 3 weeks remain relatively free from bacterial invasion, provided that they were fresh and clean when placed in the incubator. It was shown, however, that eggs which come from fowls that are permanent bacillus (*Bacterium pullorum*) carriers are often exceptions to the rule.

The carrier problem in bacillary white diarrhea of young chicks has assumed considerable importance in recent years.[2] It has been demonstrated beyond doubt that the mother hen is the permanent source of infection, as the result of acquiring the disease early in her existence, or after full maturity. The permanent seat of infection is the ovary, which in many instances becomes so greatly involved that the ova are discolored and misshapen, and the ovary presents a decidedly pathological appearance. The ova harbor the disease organism. Furthermore, ova which develop into apparently normal yolks frequently carry the organism, *Bacterium pullorum*, to the time of full formation of the egg. Infected eggs produce infected chicks, and although chicks succumb frequently before their embryonic development is completed, a large percentage of them emerge from

[1] Rettger, L. F., *Centralbl. f. Bakteriol., 2te Abt.*, 1913–14, xxxix, 611; *Bull. Storrs Agricultural Experiment Station*, 1913, No. 75.

[2] Rettger, L. F., and Stoneburn, F. H., *Bull. Storrs Agricultural Experiment Station*, 1909, No. 60; 1911, No. 68. Rettger, L. F., Kirkpatrick, W. F., and Stoneburn, F. H., *Bull. Storrs Agricultural Experiment Station*, 1912, No. 74. Rettger, L. F., Kirkpatrick, W. F., and Jones, R. E., *Bull. Storrs Agricultural Experiment Station*, 1914, No. 77.

the shell apparently sound and well. However, they soon acquire the disease and many of them fall a prey to the organism which they carried for a long time in their yolk. Those which survive often become permanent bacillus carriers, and thus the cycle of infection is completed. Furthermore, these chicks are a constant source of infection to other chicks and mature stock as well.

The occurrence of *Bacterium pullorum* in eggs has been a subject of serious consideration, but only from the standpoint of breeding and the perpetuation of sound stock. The system of testing breeding hens by the agglutination test, which is now being conducted in several of the states, marks but a culmination of the efforts that are being made to combat the disease through the detection and the elimination of the permanent carriers of bacillary white diarrhea.

There has been much speculation from time to time as to whether the presence of *Bacterium pullorum* in an egg renders it unsafe as an article of diet for man or any of the lower animals other than chicks, and especially for young children and infants. Little serious thought was given to this question, however, and it was dismissed with the assumption that such an element of danger, if it existed at all, was but very slight. While it is not the purpose of this paper to spread undue alarm, there is sufficient evidence on hand to show that *Bacterium pullorum*, when fed to some of the lower animals, may produce most serious consequences, and even death. In other words, it has been demonstrated that this organism may manifest itself as one of the group of so called "food-poisoning bacteria."

The marked pathogenicity of *Bacterium pullorum* for young chicks has been so frequently demonstrated that little need be said concerning it here. Chicks under 1 week of age are extremely susceptible to the influence of this organism, whether infection takes place through the mouth or the skin (subcutaneous injection), and the mortality is usually very high. It had long been supposed, however, that the ability of this organism to produce disease did not extend beyond young chicks.

Jones[3] was the first to demonstrate that *Bacterium pullorum* may be pathogenic for fully mature fowls. On a large poultry farm in the state of New York

[3] Jones, F. S., *Jour. Med. Research*, 1912–13, xxvii, 471.

a peculiar epidemic manifested itself in a flock of about 700 adult hens, the resultant mortality being 50. This epidemic was most clearly traced to infected eggs, as the following account will show. There had been no history of bacillary white diarrhea on the farm. A neighbor had been meeting with serious losses, however, and a number of eggs from his fowls were incubated on the farm which had hitherto been free from the disease. Nearly all the chicks that were hatched from these eggs died within 10 days from bacillary white diarrhea. The eggs which failed to hatch were inadvertently fed on Mar. 12 to the adult hens. On Mar. 28 a few of the fowls which had eaten the eggs died, and from that time on for a period of a month 50 of the hens died, with essentially the same symptoms. *Bacterium pullorum* was recovered from various internal organs of hens that were examined, as well as from chicks hatched from the neighbor's eggs, and from the ovaries of the neighbor's adult fowls.

We have obtained similar results, though on a much smaller scale. In a series of experiments conducted in 1913 on mature fowls, and in which bouillon cultures of *Bacterium pullorum* were fed to the fowls along with the regular dry mash, three deaths occurred in a pen of twelve hens. Two of the hens were sent to the laboratory for bacteriological examinations. From the liver, lungs, spleen, and heart of both these victims *Bacterium pullorum* was easily recovered, and the growths obtained on agar by direct inoculation were such as to indicate that the bacilli were present in the blood in very large numbers. Postmortem examinations did not reveal any marked or gross lesions. The deaths occurred within a period of 1 to 2 weeks after the first ingestion of the bouillon cultures. Other hens which appeared to be affected completely recovered in a relatively short time.

The first information as to the real toxicity of *Bacterium pullorum* for experimental animals was conveyed by Smith and Ten Broeck.[4] In comparative studies of the properties of this organism and of the bacillus of fowl typhoid these authors demonstrated that the bacterium-free filtrates of 5 to 15 day old bouillon cultures of *Bacterium pullorum* were decidedly toxic to full grown rabbits when given by intravenous injection. Death followed within 2 hours, or there was marked dyspnea followed by death over night or by loss of weight and subsequent recovery.

Gage[5] has shown that rabbits are very susceptible to even very small doses of *Bacterium pullorum* by subcutaneous injection, and that for this reason it is difficult to immunize rabbits to the organism.

---

[4] Smith, T., and Ten Broeck, C., *Jour. Med. Research*, 1915, xxxi, 547.

[5] Gage, personal communication.

The present investigation divides itself into two distinct phases: first, a study of the toxicity or disease-producing properties of *Bacterium pullorum* when administered orally, either with food or with the aid of a pipette; and, second, an investigation of the heat tolerance of this organism in infected eggs which are being prepared for table use by the usual processes of boiling, coddling, frying, etc.

The Toxicity of Bacterium pullorum when Introduced through the Mouth and Digestive Tract.

The experiments were conducted on 22 rabbits, 11 kittens, 11 guinea pigs, and 4 white rats. The method of administering the test organism varied. In some instances water suspensions of slant agar cultures were mixed with the regular feed, while at other times these suspensions were given with a pipette. In a number of the experiments the yolks of eggs were employed which had been artificially infected with pure cultures of the organism by injecting relatively small numbers directly into the yolk, through the shell and white, and incubated for at least 2 or 3 days. The different animals were weighed at frequent intervals, usually each day, and their general condition was observed. Those that died were subjected to postmortem examination, with a special effort to determine the presence or absence of *Bacterium pullorum* in the blood of the internal organs.

In Table I are given the results of the infection experiments with rabbits, in so far as the weights and deaths are concerned. It will be seen that the initial weights of the animals varied from 375 to 2,455 gm., the majority of the rabbits being half grown or smaller. More complete data are given in the individual records following the table.

Rabbits 1 and 2 were fed large amounts of suspensions of five different strains of *Bacterium pullorum* (1 to 5 cc.) daily. On the 5th day they refused to eat, and died on the 7th and 6th days. Pure cultures of *Bacterium pullorum* were obtained from the internal organs. Rabbits 3, 4, 5, and 6 received one large dose of the organism with the food. From the blood of each of these animals the bacterium in question was recovered in large numbers, after death. Rabbits 7 and 8 were given the surface growth of one tube of slant agar. Blood

TABLE I.

Rabbits. Weight in Gm., and Mortality Records.

Rabbit No.		Initial weight.	Days after first administration of *Bacterium pullorum*.											
			1	2	3	4	5	6	7	8	9	10	40	
1	Infected..................	380							Dead.					
2	"	375						Dead.						
3	"	650			640		550	540	"					
4	"	420	412	405	417	435	411	387	"					
5	"	475	482	483	488	452	438	Dead.						
6	"	385			380		342	337	"					
7	"	545	558	560	540	500		495	"					
8	"	435	434	432	423	405	Dead.							
9	"	615	592	630	635			550	"					
10	"	745	700	723	730			785		850		840	1,428	Alive.
11	"	648	665	690	685			675		725		770	1,334	"
12	Control..................	410	412	425	420			407		480		475	1,140	"
13	Infected..................	2,455	2,430	2,440		2,436			2,485	After 18 days			2,390	"
14	"	885	870	925	1,010	955	1,005		1,060		1,080	After 21 days	1,120	"
15	"	1,240	1,220	1,250	1,360	1,290	1,320		1,365		1,380	" 21 "	1,350	"
16	"	1,100	1,138	1,160	1,250	1,190	1,240	1,075	Dead.					
17	"	985	952	940	805	730	735	Dead.						
18	"	1,180	1,162	1,140	1,300			1,030	"					
19	"	1,370	1,362	1,370	1,325	1,370	1,335	1,215	"					
20	Control..................	1,308		1,325	1,400	1,310	1,325			1,400	Alive.			
21	"	1,210		1,230	1,285	1,240	1,255			1,290	"			
22	Infected..................	1,980	1,835	1,845		1,770	1,720	Dead.						

tests were again positive. Rabbit 9 was fed 0.1 of a slant agar growth. Death occurred on the 7th day. *Bacterium pullorum* was demonstrated in the blood.

Rabbit 10 received 0.3 of a 24 hour slant agar culture. It continued to gain weight for 40 days. It was then given 0.5 cc. of a mixture of three strains, but continued to gain for 2 weeks. A third dose of 0.7 cc. likewise had no visible effect.

Rabbit 12 served as a control animal, and was therefore not subjected to an infection experiment. Aside from this rabbit there were at all times at least two or three rabbits which belonged to the same lots as the test animals, and which were kept under practically the same conditions, except for the administration of suspensions of *Bacterium pullorum.* None of these stock rabbits died or showed any indications of illness.

Aside from a loss of 65 gm., Rabbit 13 was apparently unaffected after receiving through a pipette 2 cc. of a suspension obtained by washing three slant agar cultures.

Rabbits 14 and 15 received 0.5 and 1.0 cc. respectively of the yolk of incubated eggs which had been artificially infected with *Bacterium pullorum.* Rabbits 16, 17, 18, and 19 were given with a pipette 1.5, 2.0, 3.0, and 5.0 cc. of the yolk of artificially infected eggs. In addition to losses in weight, and death in each instance, there were indications of a diarrheal condition. With the exception of No. 18, blood tests with each of these animals for *Bacterium pullorum* gave positive results.

Rabbits 20 and 21 were kept as controls in separate cages. They remained apparently normal.

Rabbit 22 was a good sized adult male which had been suffering from a large abdominal abscess. Although the abscess was lanced it continued visibly to affect the health of the animal. 10 cc. of the yolk of an infected egg were given by mouth. On the 5th day there were marked diarrheal symptoms, and on the 6th day the rabbit died. *Bacterium pullorum* was recovered from the heart, liver, and lungs in large numbers.

Postmortem examination of the rabbits which died apparently from the effects of infection with *Bacterium pullorum* revealed no gross

lesions or other marked pathological condition. The small intestine was usually empty and decidedly pale. At times a light yellow viscid fluid was present in the lumen. In the lower intestine the contents were often less firm than in the normal rabbit, and frequently there was evidence of a diarrheal condition.

The liver was more or less congested. The surface was often marked by minute areas which appeared to be of a necrotic character. Aside from some congestion the spleen and kidneys were to all appearances normal. No pathological condition of the heart and lungs was visible to the unaided eye. Since no investigation was made of the histological structures of the different organs no details can be given as to their minute pathology.

The internal organs of the cats that died showed no marked pathological appearances, and to a large extent resembled those of the rabbits that succumbed to infection. Congestion was apparent, especially in the liver. Culture tests upon Cats 1, 2, 6, 7, and 8 gave positive results, *Bacterium pullorum* being recovered with ease from the blood of the liver, kidney, and lungs, except in No. 7 in which only the lungs contained the organism in question in sufficient numbers to obtain positive cultures. Agglutination tests with positive fowl sera proved the organism to be *Bacterium pullorum*.

The early death of the control animal was either due to some other cause or agent, aside from *Bacterium pullorum*, or was the result of rapid infection of this kitten from the vomited matter and diarrheal discharges of other kittens that were the first to be seriously affected. These experiments on kittens are to be repeated, with enough controls kept in separate cages to make the results as conclusive as possible.

White Rats.—Four white rats weighing from 100 to 250 gm. were used in these experiments. They were given one large dose of *Bacterium pullorum* in a water suspension. None of the animals showed any signs of discomfort or illness after the treatment, and all continued to increase in weight for the entire observation period of 24 days. The rats, although small as compared with adults, were apparently unaffected by one treatment with the organism (3 to 5 cc. of the suspension).

TABLE II.

Cats. Weight in Gm., and Mortality Records.

Cat No.	Initial weight.	Days after first administration of *Bacterium pullorum* by mouth. 1	2	3	4	5	6	8	10	12	14	20	23	40
1	1,088	—	1,180	1,160	—	1,192	—	1,115	1,074	985	915	790	Dead.	
		Given 2.0 cc. of suspension of *Bacterum pullorum* (with pipette) at beginning of experiment.												
2	1,098	—	1,158	1,136	—	1,200	—	1,130	997	955	890	750	"	
		Given 1.0 cc. of suspension.												
3	928	—	940	920	—	846	—	805	776	755	750	810	795	Dead. 635
		Given 0.5 cc. of suspension.												
4	618	—	632	—	—	535	492	Dead.						
		Received 0.5 cc. of suspension of *Bacterium pullorum* in water.												
5	600	—	600	—	—	590	636	535	525	530	Dead.			
		Given 1.0 cc. of suspension.												
6	590	—	590	—	—	485	492	Dead.						
		Given 1.5 cc. of suspension.												
7	660	—	680	—	—	615	620	575	595	Dead.				
		Given 2.0 cc. of suspension.												
8	620	—	820	—	—	800	710	Dead.						
		Control. Not artificially infected, but kept in same enclosure as Cats 4 to 7.												
9	990	935	910	895	885	880	—	795	770	770	685	700	710	Alive.
		Received 0.5 cc. of suspension.												
10	990	1,000	965	980	980	940	—	990	925	905	865	880	890	"
		Given 1.0 cc. of suspension.												
11	730	740	746	725	745	705	—	710	685	695	690	650	Alive.	
		Given 0.5 cc. of suspension.												

Kittens 1 to 3 were from one litter, Nos. 4 to 8 from another, and Nos. 10 and 11 from a third. While No. 8 was not artificially infected she was allowed to be in contact with Nos. 1 to 7 which were fed suspensions of *Bacterium pullorum* with a pipette. As vomiting and purging occurred very soon in these cats, the possibility of the control's becoming infected early cannot be excluded.

Kittens 4 to 7 were fed the bacterial suspensions 3 days later than Nos. 1 to 3. Signs of disturbances became apparent in both groups at about the same time. Vomiting, diarrhea, and rapid loss in weight were the most marked symptoms in the animals that died. Vomiting and diarrhea were most acute. The temperature during the time of the most severe attacks was decidedly subnormal, and for several days before death the kittens were extremely weak

Guinea Pigs. Weight in Gm., and Mortality Records.

Guinea pig No.	Initial weight.	Days after first administration of *Bacterium pullorum* by mouth, and of additional doses. 1	2	3	4	5	6	8	10	12	14	16	18	20	
1	540	—	—	560	—	540	525	528	529	525	515	510	510	538	Alive.
	Bacterial suspension of *Bacterium pullorum* mixed with food at beginning of experiment.														
2	510	—	—	512	—	502	495	482	512	498	505	518	514	555	"
	Same treatment as No. 1.														
3	717	718	710	710	700	723	702	703	—	694	710	698	705	740	"
	Same treatment as No. 1.														
4	365	370	385	—	360	340	325	Dead.							
	Given varying amounts of infected yolk daily for 6 days (1 to 5 cc. of yolk.)														
5	75	78	80	85	84	—	83	Dead.							
	Given 0.5 cc. of heavy bacterial suspension at beginning of experiment.														
6	365	370	385	360	340	325	330	275	Dead.						
	3cc.	3cc.	5cc.	8cc.	—	3cc.	8cc.	of infected yolk, given on respective dates.							
7	425	425	420	405	405	410	400	415	430	—	—	—	—	Dead.	
	7cc.	5cc.	6cc.	5cc.	4cc.	of infected yolk.									
8	315	—	310	—	330	315	—	330	—	335	—	—	—	355	"
	10cc.				10cc. of infected yolk.										
9	315	—	310	—	265	285	—	295	—	300	—	—	—	325	"
	10cc.				10cc. of infected yolk.										
10	345	320	Dead.												
	One feeding, half of an infected yolk.														
11	405	380	355	320	Dead.										
	One feeding, half of an infected yolk.														

In Guinea Pig 4 a diarrheal condition was apparent on the last 2 days. The liver and kidneys were congested. Other organs were normal. Cultural tests were negative. Guinea Pig 7 showed evidence of marked diarrhea just before death. There was some congestion of the liver and spleen, and hemorrhage in the pericardial sac. Culture tests from the different organs were negative. In Nos. 6, 7, and 10 slight diarrhea was apparent, but no other symptoms, except loss of weight and lessened appetite, were observed.

Cultural tests were again negative. *Bacterium pullorum* was isolated from the liver and lungs of Guinea Pig 11, but not from the blood of any of the other five animals that died.

DISCUSSION.

The foregoing data on the oral administration of *Bacterium pullorum* can leave no doubt as to the so called toxicity of this organism for young rabbits when given by mouth. The results of the feeding experiments with kittens of different ages are less conclusive, and require further substantiation, in view of the fact that there was no satisfactory control. They are strongly indicative, however, of a most harmful influence exerted by relatively large numbers of the organism when administered by mouth. The symptoms of the disturbance produced in the kittens were those of a food-poisoning organism, especially the vomiting and diarrhea, and the extreme emaciation. The possibility that these symptoms are due to a complication of disturbing factors, cannot be entirely ignored, however.

Adult guinea pigs are but slightly susceptible to *Bacterium pullorum*, when ingested, though six of the eleven animals employed died, some early and others late in the periods of observation. With but one or two exceptions, they were given large amounts of the organism. Further experiments with guinea pigs are now in progress. Rats, according to the above data, are immune to disturbing influences of *Bacterium pullorum* when it is given by the mouth, even in large numbers.

Although there are no cases on record of food-poisoning, enteritis, or other ailments in man which have been ascribed to *Bacterium pullorum*, the possibility of danger from infection with this organism can no longer be ignored, especially in so far as invalids and young children are concerned. Eggs are among the most common articles of diet, and for the sick and convalescent as well as for children of all ages they often rank with milk as one of the most important foods. Not only are they consumed soft boiled, but they are frequently prescribed in the raw state.

Fresh eggs, when they are infected with *Bacterium pullorum*, contain this organism in such small numbers as to constitute no real danger of disturbance even for the most infirm, or for the smallest infants. It has been conclusively demonstrated, however, that the organism multiplies very rapidly in the yolks of infected eggs, when

the eggs are held at or near so called incubator temperature.[6] Hence, eggs which are left in the nest under broody hens for but comparatively few hours, or which are not kept in cool places during storage and transportation, especially during the warm summer months, harbor the organism in large numbers. In fact, they are so abundant that the inoculation of the surface of slant agar with but a small portion of the yolk held in a platinum loop results in the production of an almost solid surface growth on the agar.

It has also been shown that in various sections of this country a large percentage of the flocks and of the individual hens are permanent carriers of white diarrhea (*Bacterium pullorum*) infection. In the survey of the conditions in Connecticut it was found that out of 107 flocks which were tested for ovarian infection with *Bacterium pullorum* 79, or 74 per cent, possessed bacillus carriers, and of a total of 13,831 fowls that were subjected to the agglutination test 1,417, or 10.24 per cent, gave positive indications of ovarian infection.[7] In an investigation recently conducted in Massachusetts similar results were obtained.[8] In some flocks over 50 per cent of the individual fowls that were tested were reactors. The same condition undoubtedly prevails throughout a large part of the Country, though no definite figures have been obtained outside of Connecticut and Massachusetts.

The common methods of boiling or frying infected eggs for the table do not necessarily render the eggs sterile, in so far as *Bacterium pullorum* is concerned, as the following data will show.

The Survival of Bacterium pullorum in the Yolks of Eggs after Various Methods of Treatment with Heat. The Influence of Cooking.

These experiments were carried on with eggs which were artificially infected with *Bacterium pullorum* in the following manner. Fresh

[6] Rettger and Stoneburn, *Bull. Storrs Agricultural Experiment Station*, 1911, No. 68. Rettger, Kirkpatrick, and Jones, *Bull. Storrs Agricultural Experiment Station*, 1914, No. 77. Rettger, *Centralbl. f. Bakteriol., 2te Abt.*, 1913–14, xxxix, 611.

[7] Rettger, Kirkpatrick, and Jones, *Bull. Storrs Agricultural Experiment Station*, in press.

[8] Gage, G. E., and Paige, B. H., *Bull. Massachusetts Agricultural Experiment Station*, 1915, No. 163.

eggs were immersed for a few minutes in alcohol, and one of the ends was flamed. A small hole was made through the shell without injuring the shell membrane. The eggs were then inoculated by injecting about 0.25 cc. of a water suspension of the organism with a sterile hypodermic syringe directly into the yolk. The hole was sealed with collodion and the eggs were incubated for varying lengths of time, usually 3 to 5 days.

Infected eggs were placed in boiling water, and held there for different lengths of time. They were then chilled in cold water and opened aseptically. Small amounts of yolk were streaked over the surface of slant agar and the tubes incubated at 37°C. The results are given in Table IV.

TABLE IV.

Time of boiling.	Coagulation of white.	Thickening of yolk.	Growths of *Bacterium pullorum* on slant agar.
min.			
1	0	0	+
2	0	0	+
2½	Slight.	0	0 + + + + +
3	+	Slight.	0 0 0 + + + +
3½	+	+	0 0 0 0 0 0 + + + +
4	+	+	0 0 0 + +

In the last column 0 indicates no growth, and + the characteristic growth of *Bacterium pullorum*. Each 0 and + sign represents an individual egg. The variation in the results of the different eggs is undoubtedly due to differences in the size of the eggs and the thickness of the shells, and perhaps in part to the differences in the numbers of bacteria in the eggs.

Poaching the eggs for ½ to 4 minutes rendered them sterile. Artificially infected eggs that were scrambled were likewise found to contain no viable organisms. On the other hand, fried and coddled eggs gave varied results, as will be seen in the following brief summaries.

In all the foregoing experiments control tests were made with eggs that had been inoculated and incubated in the same way as the others. No difficulty was experienced in obtaining an abundance of the bacilli in the yolks after the various periods of incubation of the eggs.

It should be stated that all these experiments were conducted at an altitude of 700 feet above sea level, where the boiling point of water is 99.2°C.

TABLE V.

The Effect of Frying.

Physical character of heated eggs.	Growth on slant agar.
Frying on one side only.	
Soft.	+ + + +
Medium.	+ +
Hard.	+
Frying on both sides.	
Soft.	0 0
Medium.	0 0
Hard.	0 0

TABLE VI.

The Effect of Coddling.

Time of heating.	Growth on slant agar.
min.	
3	0 +
3½	0 0
4	0 + + +
4½	+
5	0 0
7	0
10	0

The eggs were coddled by pouring boiling water over them in a granite receptacle and allowing them to stand away from a flame or stove for the different lengths of time. About 1 quart of water was employed for each egg.

The experiments on the viability of *Bacterium pullorum* in egg yolk after varying periods of heating show that the organism under the stated conditions possesses a high degree of resistance. Even boil-

ing for 4 minutes did not in every instance destroy it. This resistance is due undoubtedly to the peculiar protection which is afforded, first by the shell, then by the egg white, and finally by the yolk itself. The high per cent of fat in the yolk is undoubtedly an important factor.

It has been demonstrated by Smith[9] and others that tubercle bacilli are less readily killed by the ordinary process of pasteurization when they are held in the film or pellicle layer of milk than in the whole or mixed milk.

Other organisms of the colon-paratyphoid-typhoid group would undoubtedly show the same resistance under similar conditions of environment. Chantemesse and Rodriguez[10] report an epidemic of food-poisoning which was due to cream cakes. The meringue of the cakes was found to contain a peculiarly toxic microorganism of the *Bacillus paratyphi* type, and although the meringue had been heated to browning numerous bacilli of this type were found in the interior portion. Quite recently a small epidemic of typhoid fever was pronounced to have been caused by the consumption of baked spaghetti.[11] The typhoid bacillus was readily identified in the inner portion of spaghetti that was artificially inoculated and baked, in spite of the fact that the dish was subjected to sufficient heat to brown the surface and to char the protruding ends of the spaghetti sticks.

SUMMARY AND CONCLUSIONS.

The problem of eradicating ovarian infection in the domestic fowl assumes still greater importance than heretofore, in the light of data recently acquired. Not only is it of great significance to eliminate the permanent carriers of *Bacterium pullorum* from all flocks of fowls from the standpoint of successful poultry breeding, but also because they constitute a possible source of danger to man.

Eggs which harbor *Bacterium pullorum* in the yolk in large numbers may produce abnormal conditions, when fed, not only in young chicks, but in adult fowls, young rabbits, guinea pigs, and kittens. The toxicity for young rabbits is most pronounced, the infection usually resulting in the death of the animals. In kittens the most prominent symptoms are those of severe food-poisoning with members of the paratyphoid group of bacteria. The possibility of infected eggs causing serious disturbances in young children and in the sick

[9] Smith, T., *Jour. Exper. Med.*, 1899, iv, 217.

[10] Chantemesse and Rodriguez, *Bull. Acad. de méd.*, 1914, lxxi, 245; Abstract in *Experiment Station Record*, 1914, xxxi, 555.

[11] Johnston, H., *Health News*, 1915, xxxi, 173.

and convalescent of all ages must therefore receive serious consideration.

Ovarian infection of fowls is very common throughout this country. Hence, a large proportion of the marketed eggs are infected with *Bacterium pullorum*. When such eggs are allowed to remain in nests under broody hens, or in warm storage places, for comparatively few hours, they contain large numbers of the organism.

Soft boiling, coddling, and frying on one side only do not necessarily render the yolks free from viable bacteria; therefore, eggs which have gone through these processes may, like raw eggs, be the cause of serious disturbances in persons who are particularly susceptible to such influences, and especially to infants.

That no well authenticated instances of egg-poisoning of this kind are on record does not warrant the assumption that there have been no cases. The etiology of infantile stomach and intestinal disturbances is as yet too little understood; in fact, it may be said that many of these disorders have no known cause, and almost as much may be said regarding gastro-intestinal diseases in later life. Furthermore, since the ailments caused by infected eggs would not make themselves felt presumably until several days after their ingestion, little or no suspicion would fall upon the eggs. It may be said, too, that the wide distribution of ovarian infection in the domestic fowl has come about only in the last few years, hence its possible danger to man is one of recent development.

SERUM CHANGES AND THE CAUSE OF DEATH IN EXPERIMENTAL PANCREATITIS.

Studies on Ferment Action. XXX.

By WILLIAM PETERSEN, M.D., JAMES W. JOBLING, M.D., and A. A. EGGSTEIN, M.D.

(*From the Department of Pathology, Medical Department, Vanderbilt University, Nashville.*)

(Received for publication, January 26, 1916.)

Incidental to a study of the ferment balance of the serum during various pathological conditions we have had occasion to observe the serum changes in a series of eighteen dogs in which an acute experimental pancreatitis had been produced.

Various theories have been advanced to account for the marked intoxication resulting from an acute pancreatitis. Opie (1) in this country, and Eppinger (2), Pólya (3), von Bergmann and Guleke (4), and numerous other workers in Europe have fully discussed the problem. The changes in the lipase titer have been reported by Whipple and Goodpasture (5).

Most workers are convinced that an activation (probably intracellular) of the tryptic proenzyme occurs with a resulting intoxication of the animal because of the sudden formation and absorption of toxic split products. Coincident with this change there may occur a saponification of fats due to the simultaneous activation of the pancreatic lipase with a further injury to the pancreatic tissue by the soaps so formed. The means of activation of these ferments, resulting from a primary injury, may, of course, be diverse: bacterial infection, either through the ducts or lymphatics; mechanical blocking of the ducts and activation from bile; or activation from enterokinase and from tissue juice resulting from direct trauma, possibly from injury following vascular changes. While, therefore, the primary factor may be diverse, the resulting pathological lesions and the cause of death are uniform.

In the following illustrative experiments three different substances were injected as activators: bile salts, as first recommended by Flexner (6), active trypsin solutions, and sodium oleate solutions.

These experiments and the charts of the serum changes follow.

Dog 1.—Weight 4.5 kilos. 0.2 gm. of bile salts was injected into the pancreatic duct with subsequent ligation, at 9.30 a.m. on May 10, 1915. Serum samples were collected before the operation, at 10 and 11 a.m., and at 1 and 3 p.m. on each of the following 2 days and after about 3 weeks (June 3, 1915). The serum changes are shown in detail in Text-fig. 1.

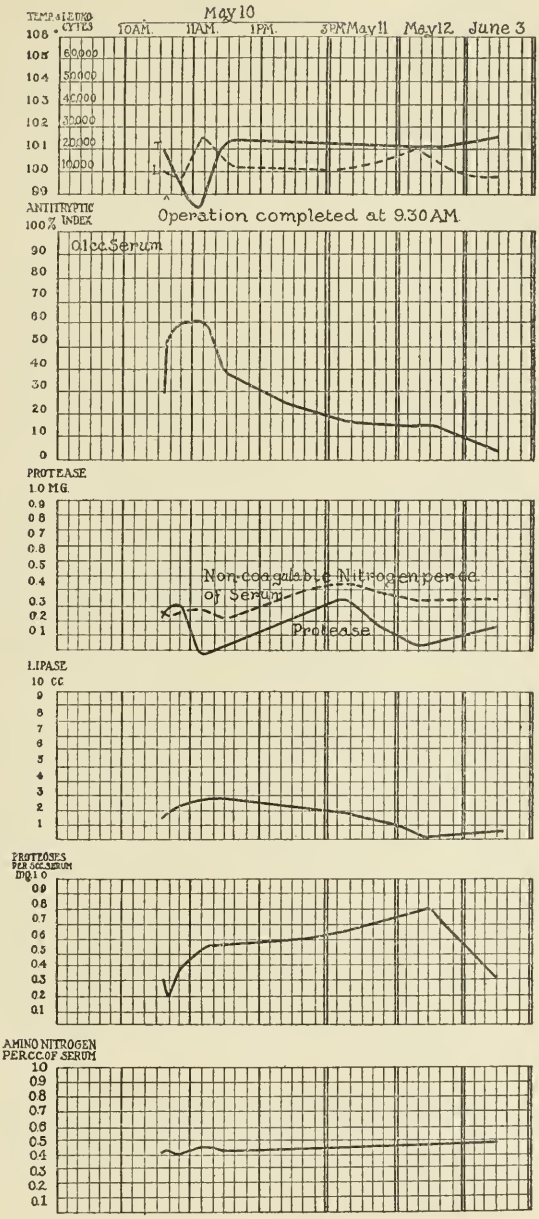
The animal was killed on June 3, 1915. The pathological changes noted at autopsy were as follows: The pancreatic duct was occluded. The body of the pancreas was atrophic, fibrous, with large areas of fatty change and small necrotic foci containing caseous material. The tail of the pancreas showed less change. The viscera presented no pathological alteration other than fatty changes.

Dog 2.—Weight 5 kilos. 1 gm. of sodium oleate was injected into the pancreatic duct with subsequent ligation, at 11.30 a.m. (May 14, 1915). Serum samples were collected before the operation at 11.50 a.m., and at 1.30 and 3.30 p.m. on the 3 succeeding days, and after several weeks (June 4, 1915). The dog was killed after being used in another experiment. The serum changes are shown in detail in Text-fig. 2. The findings at autopsy were as follows: The pancreatic tissue was represented by a small remnant about one-fourth the size of the original tissue, rather firm, and pale yellow in color. The duct was occluded and there were numerous firm adhesions to the adjoining viscera. Scattered throughout the remnants of the gland were small more or less caseous areas of necrosis.

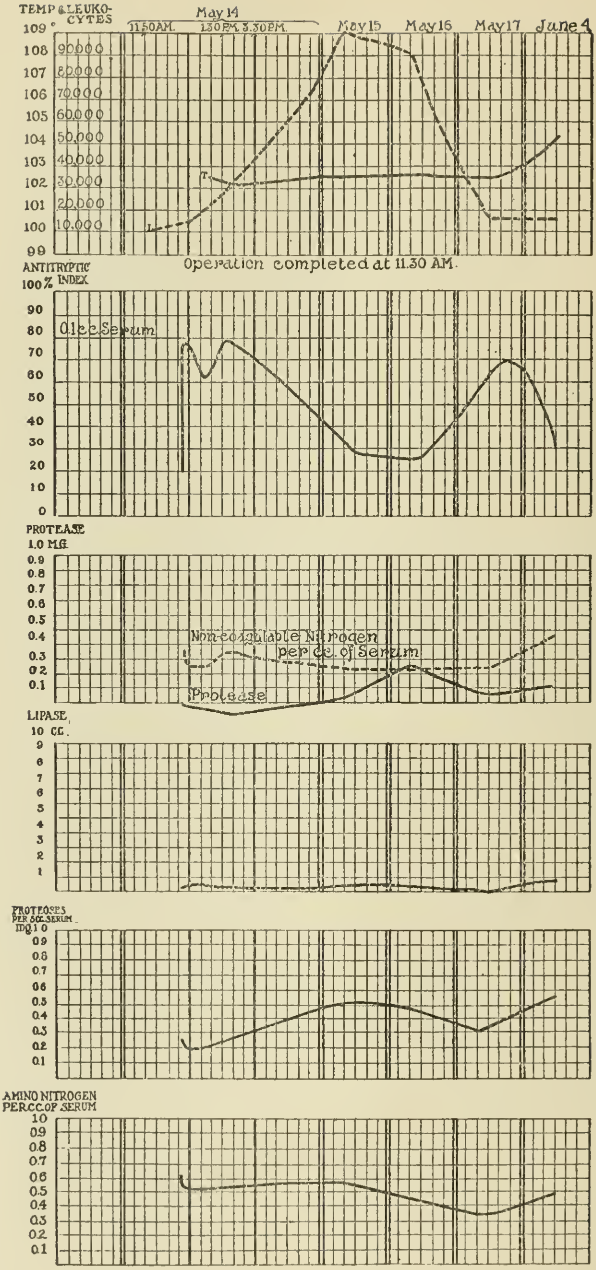
Dog 3.—Weight 5.1 kilos. 0.2 gm. of trypsin (purified) was injected into the pancreatic duct, with subsequent ligation, at 9.10 a.m. (June 7, 1915). Serum samples were collected before the operation at 9.45 a.m., noon, and 3 p.m., and at 8.30 a.m. and 2 p.m. the following day. The animal died at 2.15 p.m. (June 8, 1915). The serum changes are shown in detail in Text-fig. 3.

The autopsy findings were as follows: On opening the abdominal cavity a considerable amount of sterile, hemorrhagic, opaque fluid was found. This exudate contained 0.28 mg. of non-coagulable nitrogen per cc., and had a slight antitryptic action. Incubated under toluol the non-coagulable nitrogen increased 0.27 mg., and under chloroform 1.72 mg. in 16 hours, indicating the presence of considerable amounts of proteolytic ferments. The pancreatic tissue was hemorrhagic and contained numerous areas of necrosis throughout; there were numerous areas of fat necrosis scattered over the omentum. Fibrinous adhesions were found about the pancreas and adjacent viscera.

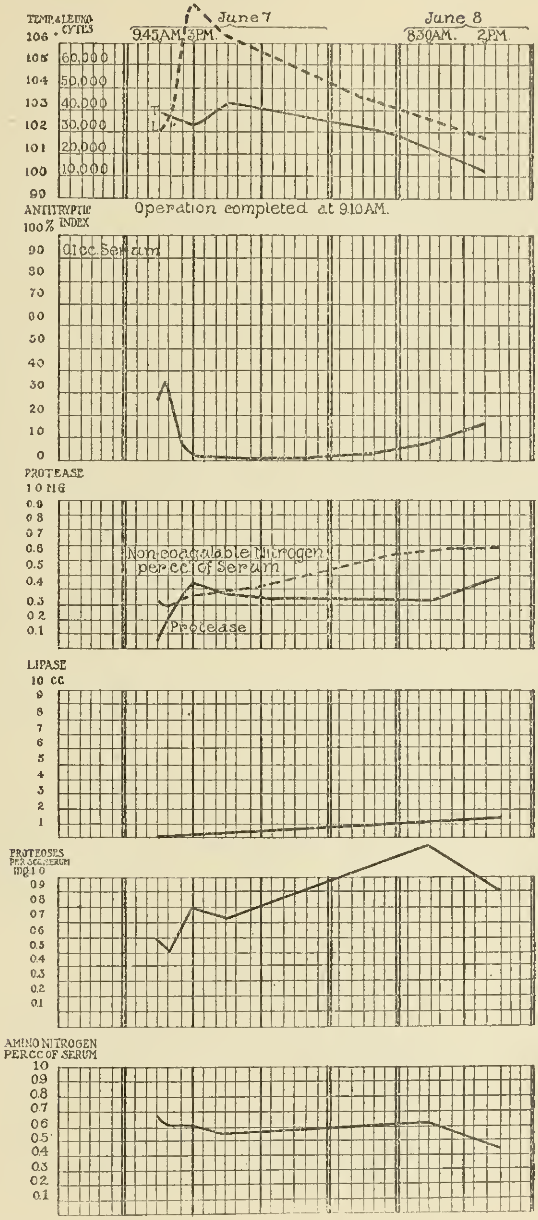
In the first animal the injection of bile salts caused only a moderate pancreatitis, although at the time the abdomen was closed after the injection a thorough infiltration of the pancreatic tissue was noted.



TEXT-FIG. 1. Serum changes accompanying acute pancreatitis due to bile salt injections.



TEXT-FIG. 2. Serum changes accompanying acute pancreatitis due to soap injections



TEXT-FIG. 3. Serum changes accompanying acute pancreatitis due to trypsin injections.

No increase in temperature resulted; only a moderate leukocytosis and that only for a short period of time. Immediately after the operation the antiferment increased while the protease decreased; the balance was restored to a normal level the following day. The serum lipase increased slightly. There was noted a marked increase in serum proteoses during the period following the operation; after 3 weeks a normal value was again found. The amino nitrogen in this, as in the other animals, showed only very slight changes.

In the third dog the injection of the trypsin resulted in a rapidly fatal intoxication and it will be observed that the serum changes were different. There was a marked leukocytosis—80,000—as an index of the intoxication, with a progressive decrease in temperature. The antiferment, after a short rise, declined progressively, while the serum protease increased and remained high. The proteoses, after a slight initial decrease, frequently observed in the period following the mobilization of protease, increased markedly. There was only a slight change in the lipase.

The picture closely resembles the condition found in true trypsin shock (7), except in the behavior of the serum lipase. In conjunction with Experiment 2 it would seem that the decrease in the antiferment and the increase in protease have had some relation to the fatal outcome in this case.

In the second dog in which soap was injected, we find the reverse of the preceding changes, as might be expected from the difference in the action of the substances. The tissue destruction was quite marked and the whole pancreas even before closing the abdomen presented a deeply engorged, hemorrhagic, semitranslucent mass almost twice the normal size. It will be observed that the leukocyte count was also high—100,000 after 24 hours. Despite the severity of the local process the serum changes were slight and the animal showed little evidence of a general intoxication. The antiferment increased immediately, but the serum picture was practically unaltered after 24 hours. The lipase remained low. As contrasted with the two previous animals it will be seen that the increase in proteoses was not only delayed but actually less in amount.

DISCUSSION.

From the series of animals, of which these three are representative, we believe that we are justified in assuming that death is due to the sudden flooding of the blood stream with the higher split products formed at the expense of the pancreatic tissue, of which the proteose increase is an index. Except in the experiments in which active trypsin is used for injection there is no increase in serum protease at any time, as would be expected if the intoxication were a true trypsin shock, nor is there much change in serum lipase (esterase), the condition in this respect resembling closely the results observed following the injection of protein split products (8).

Here, as in other protein intoxications (9) the increase in antiferment seems to be of distinct value in the protection of the animal; in the experiment in which soap was used for the injection this becomes apparent. The increase in antiferment was marked, while the delay in the digestion of the pancreas and the consequent lessening of the shock is indicated by the gradual and relatively small increase in the amount of proteoses present in the serum.

In view of Whipple's results concerning the non-toxicity of the abdominal exudate (5) and our own experiments, it would seem that the only beneficial effects which might possibly have accrued from operative interference in cases of human pancreatitis have resulted from the prolonged anesthesia rather than from the surgical drainage. The inhibitory effect of anesthetics on the development of toxicity for protein intoxication is well known.

CONCLUSIONS.

1. The serum changes observed during acute experimental pancreatitis indicate that the shock and death are due to an intoxication from protein split products, and not to an intoxication from pure tryptic ferment.

2. When the pancreatitis is produced by the injection of an antiproteolytic substance (sodium oleate), the degree of intoxication bears no relation to the degree of tissue destruction.

3. The increase in serum antiferment apparently favors the recovery of the animal.

BIBLIOGRAPHY.

1. Opie, E., Disease of the Pancreas, Its Cause and Nature, 2nd edition, Philadelphia and London, 1910.
2. Eppinger, H., *Ztschr. f. exper. Path. u. Therap.*, 1905–06, ii, 216.
3. Pólya, E., *Arch. f. d. ges. Physiol.*, 1908, cxxi, 483.
4. von Bergmann, G., and Guleke, N., *München. med. Wchnschr.*, 1910, lvii, 1673.
5. Whipple, G. H., and Goodpasture, E. W., *Surg., Gynec. and Obst.*, 1913, xvii, 541.
6. Flexner, S., *Jour. Exper. Med.*, 1906, viii, 167.
7. Jobling, J. W., Petersen, W., and Eggstein, A. A., *Jour. Exper. Med.*, 1915, xxii, 141.
8. Jobling, J. W., Petersen, W., and Eggstein, A. A., *Jour. Exper. Med.*, 1915, xxii, 597.
9. Jobling, J. W., Petersen, W., and Eggstein, A. A., *Jour. Exper. Med.*, 1914, xx, 468.

SERUM CHANGES FOLLOWING THYROPARATHYROIDECTOMY.

Studies on Ferment Action. XXXI.

By WILLIAM PETERSEN, M.D., JAMES W. JOBLING, M.D., and A. A. EGGSTEIN, M.D.

(*From the Department of Pathology, Medical Department, Vanderbilt University, Nashville.*)

(Received for publication, January 26, 1916.)

As a result of the work of MacCallum and his coworkers (1), our knowledge concerning disturbances of the inorganic constituents of the blood serum particularly, following the removal of the parathyroids has been greatly advanced, and the antagonistic effect of the calcium ions established.

Certain observations have accumulated, largely of a clinical nature, which seem to indicate that in the tetany of infancy dietary factors are of considerable importance both from an etiological and therapeutic point of view. The spasmophilic infant rapidly recovers on a proper diet, and the evidence seems to indicate that phosphorus and certain of the unsaturated oils are of aid in expediting a favorable outcome.

In adults, too, we have to consider the peculiar form of tetany associated with gastric and occasionally intestinal disturbances, under conditions, therefore, which may be considered to offer opportunities for the absorption of the higher split products of proteins, the gastric digestion, of course, only splitting through to the peptones.

Experimentally the observation has been frequently recorded that the feeding of meats is particularly prone to lead to the onset of tetany in parathyroidectomized dogs.

Considerations such as these probably led Kling (2) to study the electric excitability of animals during anaphylaxis, in an effort to correlate the increased irritability of tetany with an anaphylactic state depending on a protein intoxication.

Our knowledge concerning the pathological effects of a faulty absorption of the higher split products of proteins from the gastro-intestinal tract is as yet fragmentary, despite the obvious clinical evidence pointing in this direction. It has been observed, however, that in gastric tetany a gastro-enterostomy usually relieves the condition, the operative interference probably enabling a more rapid emptying of the stomach to the intestine with an augmented splitting of the protein contents to lower stages. Kaufmann's work is of particular interest in this connection (3).

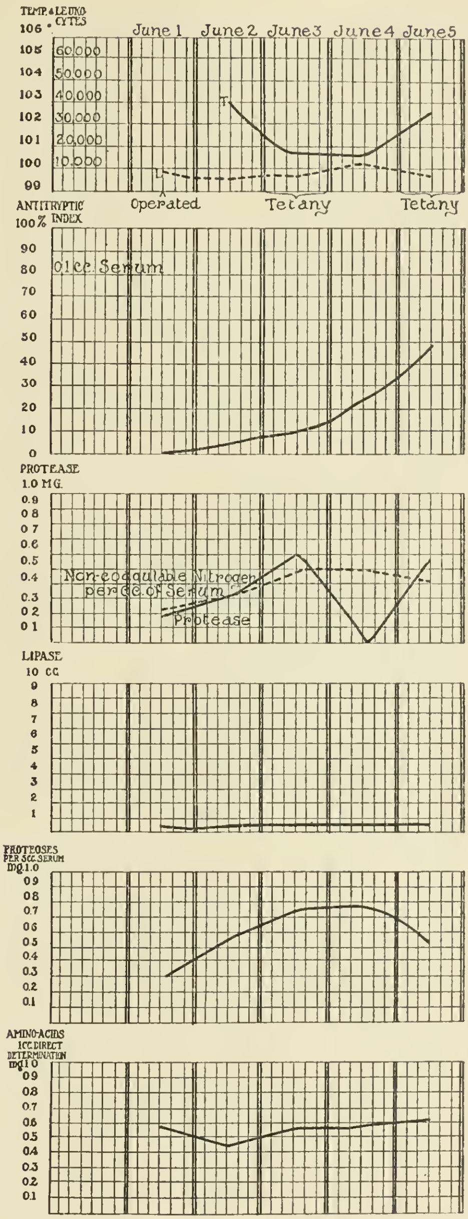
In the few cases of true intestinal tetany that have been reported, there is considerable evidence that an achylia gastrica frequently complicates the picture, which again would offer every opportunity for a rapid absorption of the higher split products from the intestine, which under normal conditions is only afforded an opportunity to absorb the lower split products (Fleiner (4), Quosig (5)). The relation of tetany in infants is of particular interest in this connection; for substances which tend to increase the antiferment titer—unsaturated fats, phosphorus—have on empiric grounds been employed in the therapy of spasmophilia for many years, indicating that a suppression of a possibly faulty splitting of proteins may form the basis for this theory. Our interest in experimental tetany has centered chiefly, therefore, in a study of the changes of the serum and the relative amounts of split products therein contained.

The experiments were made on dogs. Thyroparathyroidectomy was performed in fifteen animals, and the serum changes were studied. The following two experiments are typical of the relations noted.

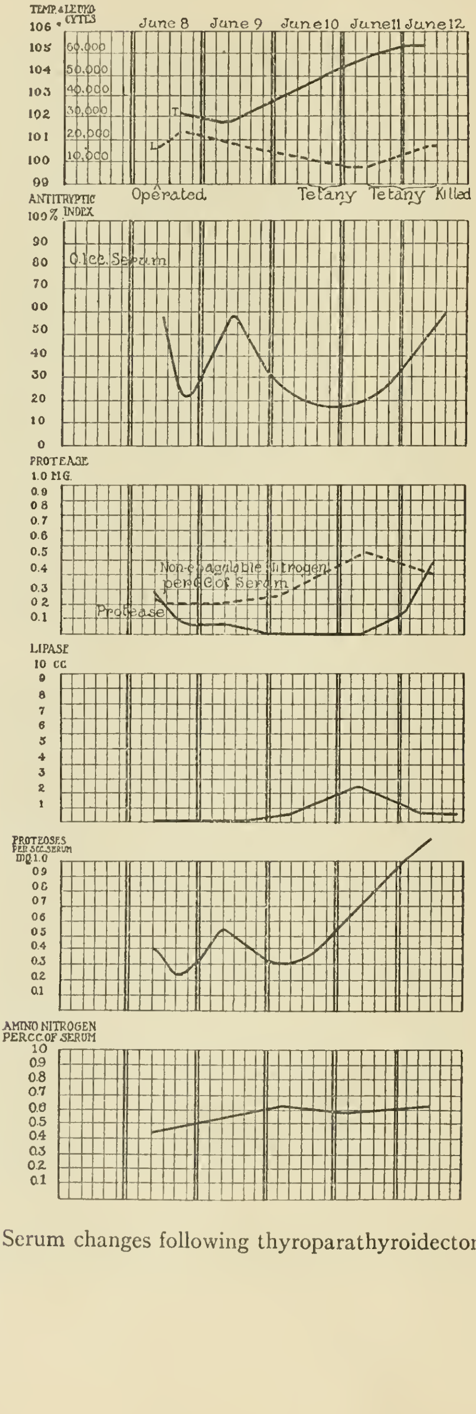
Dog 1.—Weight 5 kilos. Complete thyroparathyroidectomy on June 1, 1915. Tetany was observed on the 3rd day following the operation; on the 4th day the dog showed no symptoms; but on the next day (June 5, 1915) there was marked tetany. The animal was found dead the following morning. In Text-fig. 1 the serum changes are illustrated in detail.

Dog 2.—Weight 8 kilos. Complete thyroparathyroidectomy on June 8, 1915. Tetany was noted in the afternoon of June 10, 1915, again in the following afternoon, and the next morning (June 12, 1915), when the animal was killed. The serum changes are shown in Text-fig. 2.

In the first dog there will be noted a gradual increase in the antiferment titer until the time of death, with an irregular protease curve.



TEXT-FIG. 1. Serum changes following thyroparathyroidectomy in Dog 1.



TEXT-FIG. 2. Serum changes following thyroparathyroidectomy in Dog. 2.

The maximum protease activity was noted in the animal during the time when the tetany was most apparent. The non-coagulable nitrogen of the serum increased to more than twice the original amount. The lipase remained constantly low. The proteoses increased markedly. The amino nitrogen of the serum in this animal showed no change except an initial decrease.

In Experiment 2 (Dog 2) the conditions are different. The antiferment titer showed marked fluctuation, the first decline appearing shortly after the operation. The protease remained low until the last day, but the non-coagulable nitrogen increased as in the previous animal; the proteoses also accumulated during the period of tetany. The increase in amino-acids is similar to that observed in practically all the other animals during tetany. This is the only animal of the entire series in which a rise in the lipase titer was observed.

DISCUSSION.

It is apparent that the ferment changes in these animals are not in themselves related to the onset of tetany, because the protease and lipase titers, as well as the antiferment index, have no constant relation at any time. It is equally apparent that a considerable accumulation of non-coagulable nitrogen occurs in the serum long before the onset of the actual tetany.

Thus an average of all the dogs examined gives the following figures for the non-coagulable nitrogen per cc. of serum:

Before operation.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
0.21 mg.	0.31 mg.	0.39 mg.	0.50 mg.	0.44 mg.

The average proteose content per 5 cc. of serum was as follows:

Before operation.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
0.41 mg.	0.50 mg.	0.58 mg.	0.74 mg.	0.84 mg.

In four animals of the series the onset and recovery from tetany followed at equal time intervals, and the amino-acid content of the serum per cc. has been averaged under comparable conditions.

Before operation.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
0.48 mg.	0.48 mg.	0.59 mg.	0.54 mg.	0.59 mg.
Operated.	No tetany.	Tetany.	No tetany.	Tetany.

Some observers, among them Morel (6), consider the pathological effect of the parathyroidectomy due to an auto-intoxication, regarding the tetany as a secondary character. An increased nitrogenous metabolism following the removal of the parathyroids is well recognized, but it is difficult to obviate the effect of the tremendous muscular stimulation in the interpretation of the result. It is interesting to note that Falta and Kahn (7) noted an increase in the peptide nitrogen of the urine in their animals, a result possibly of the large increase in the serum which we have observed.

Whether or not the alteration in the nitrogenous constituents indicated by the serum and urine changes is wholly the result of the increased muscular activity due to the increased irritability of the nervous system, or whether the change in these constituents from a quantitative and possibly a qualitative point of view is the cause of the increase of irritability of the nervous system, is, of course, not determined in these experiments.

During the course of the work it was observed that despite the marked fluctuation of the serum protease the titer of the serum lipase remained constantly at a very low level. Stuber and Heim (8) have previously expressed the opinion that the titer of the serum lipase (esterase) was at least partially under the control of the glands of internal secretion. In a series of dogs we have injected dried typhoid bacteria (10 to 20 mg.), which normally cause a prompt and not inconsiderable mobilization of serum lipase, into the circulation of thyroparathyroidectomized dogs and into dogs after complete parathyroid and partial thyroid removal. In the majority of instances the mobilization of lipase has been less constant and of less magnitude than in normal dogs, but the experiments are not sufficiently clear cut to warrant the conclusion of a definite relation between the glandular function and the lipase titer.

CONCLUSIONS.

1. In thyroparathyroidectomized dogs the onset of tetany bears no constant relation to the ferment-antiferment balance of the serum.

2. The serum lipase titer remains at a low level throughout.

3. A progressive increase in non-coagulable nitrogen and proteoses is observed in the serum following the removal of the glands.

4. The amino nitrogen of the serum is usually increased at the time when tetany is most marked.

BIBLIOGRAPHY.

1. MacCallum, W. G., Thomson, H. S., and Murphy, Jas. B., *Bull. Johns Hopkins Hosp.*, 1907, xviii, 333. MacCallum, W. G., and Voegtlin, C., *Jour. Exper. Med.*, 1909, xi, 118. MacCallum, W. G., and Vogel, K. M., *Jour. Exper. Med.*, 1913, xviii, 618.
2. Kling, C. A., *Ztschr. f. Immunitätsforsch., Orig.*, 1912, xiii, 43.
3. Kaufmann, J., *Am. Jour. Med. Sc.*, 1904, cxxvii, 646.
4. Fleiner, W., *München. med. Wchnschr.*, 1903, l, 409.
5. Quosig, K., *München. med. Wchnschr.*, 1906, liii, 457.
6. Morel, L., *Jour. de physiol. et de path. gén.*, 1911, xiii, 542; *Compt. rend. Soc. de biol.*, 1910, lxviii, 163; 1909, lxvii, 780; 1911, lxx, 749.
7. Falta, W., and Kahn, F., *Ztschr. f. klin. Med.*, 1912, lxxiv, 108.
8. Stuber, B., and Heim, R., *München. med. Wchnschr.*, 1914, lxi, 1661.

THE DISAPPEARANCE OF DEXTROSE FROM THE BLOOD AFTER INTRAVENOUS INJECTION.

By ISRAEL S. KLEINER, Ph.D.

(*From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 27, 1916.)

HISTORICAL.

Claude Bernard[1] was the first to study the elimination of sugars after intravenous administration. He, as well as others,[2] demonstrated that dextrose, lactose, or saccharose, when thus injected in large quantity, is eliminated in the urine. Although working only qualitatively, he[3] realized that dextrose was better assimilated under these circumstances than saccharose or lactose. The time relations were worked out, particularly by von Becker, who found after injecting 1.5 gm. of dextrose into rabbits that sugar elimination by the kidneys began promptly and continued for 5 or 6 hours. Limpert and Falck[4] did the first quantitative work. Dogs of 5 or 6 kilos when given 5 to 7 gm. of dextrose intravenously excreted only traces in the urine. Larger amounts led to a slightly increased excretion. Thus it was shown early that after intravenous injection fairly large amounts of dextrose can be retained. The proportion excreted in the urine, according to von Brasol[5] and others[6] bears no constant relation to the injected dextrose; nor can the duration of the glycosuria be predicted. Von Brasol also found that the degree and duration of glycosuria were not always the same for

---

[1] Bernard, C., Dissertation, Neue Funktion der Leber als zuckerbereitendes Organ des Menschen und der Thiere, Würzburg, 1853.

[2] Kersting, *Jour. f. prakt. Chem.*, 1844, xxxiii, 58. Baumert, M., *Jour. f. prakt. Chem.*, 1851, liv, 357. Uhle, Inaugural Dissertation, Leipzig, 1852. von Becker, F. J., *Ztschr. f. wissensch. Zool.*, 1854, v, 123. All quoted by Limpert, L., and Falck, C. P., *Virchows Arch. f. path. Anat.*, 1856, ix, 56.

[3] Bernard, C., *Compt. rend. Acad.*, 1846, xxii, 536.

[4] Limpert, L., and Falck, C. P., *Virchows Arch. f. path. Anat.*, 1856, ix, 56.

[5] von Brasol, L., *Arch. f. Physiol.*, 1884, 211.

[6] Pavy, F. W., *Jour. Physiol.*, 1899, xxiv, 479. Weyert, F., *Arch. f. Physiol.*, 1891, 187. Lilienfeld, C., *Ztschr. f. diätet. u. physik. Therap.*, 1898–99, ii, 209. Wilenko, G. G., *Arch. f. exper. Path. u. Pharm.*, 1911, lxvi, 143. Bang, I., Der Blutzucker, Wiesbaden, 1913, 76.

a given animal. Gilbert and Carnot,[7] however, maintained that within certain limits the proportion of sugar eliminated is constant for each animal, and Blumenthal[8] found that the "assimilation limit;" *i.e.*, the amount which can be injected intravenously without causing glycosuria, is practically invariable for each individual. The velocity of injection plays a part, for Doyon and Dufourt[9] found that more is excreted after rapid than after slow injection. According to them, age, state of nutrition, ligation of the bile duct, and administration of alcohol are apparently without influence. Bang[10] has shown that the glycosuria and glycemia do not run parallel. The concentration of the injected sugar probably plays a part, for Wilenko[11] states that concentrated dextrose solutions (40 per cent) produce the same changes in renal permeability as concentrated salt solutions; that is, first an increased and then a decreased permeability for sugar.

During the injection of dextrose the blood sugar rises and afterwards falls, until it finally assumes a normal or subnormal figure. Von Brasol[5] injected from 0.9 to 5.3 gm. per kilo of body weight in 4 to 6 minutes. Analysis indicated that in the first 2 minutes following the injection some of the sugar had already left the blood; but no direct ratio existed between the amount injected and the blood sugar percentage. After 2 hours the sugar content of the blood was usually normal; with smaller doses (0.9 and 2.3 gm. per kilo) this level was sometimes reached in 1 hour. Butte[12] injected 3 to 10 gm. of dextrose per kilo intravenously and noted the rapid fall of blood sugar to or nearly to the normal level. With 4 gm. per kilo for example, the percentage of sugar in the blood 1¼ hours after the injection was 0.353 per cent, and 2 hours after, 0.220 per cent. In no case did he find a subnormal figure as did Grèhant[13] who found 0.036 per cent blood dextrose 2 hours after an injection of 6 gm. of dextrose per kilo. Pavy[14] found in rabbits that the blood sugar fell rapidly after an intravenous injection; for example, immediately after 4 gm. per kilo had been injected, the blood contained 1.4 per cent sugar; 5 minutes after, 1 per cent; and 15 minutes after, 0.8 per cent. Lépine[15] found that after the injection of 1 gm. of dextrose per kilo slowly into dogs, the blood sugar fell in the course of an hour below 0.1 per cent. Bang[16] injected 1 to 2 gm. per kilo intravenously and found that the maximum blood sugar was reached 2 to 5 minutes after the injection and that the high percentage was maintained for about 30 minutes, after which a rapid fall to normal or below

---

7 Gilbert and Carnot, *Compt. rend. Soc. de biol.*, 1898, v, 330.

8 Blumenthal, F., *Beitr. z. chem. Phys. u. Path.*, 1905, vi, 329.

9 Doyon, M., and Dufourt, E., *Jour. de physiol. et de path. gén.*, 1901, iii, 703.

10 Bang, I., Der Blutzucker, Wiesbaden, 1913, 77.

11 Wilenko, G. G., *Arch. f. exper. Path. u. Pharm.*, 1911, lxvi, 143.

12 Butte, L., *Compt. rend. Soc. de biol.*, 1896, iii, 274.

13 Grèhant, quoted by Butte, L., *Compt. rend. Soc. de biol.*, 1896, iii, 274.

14 Pavy, F. W., *Jour. Physiol.*, 1899, xxiv, 479.

15 Lépine, R., Le diabète sucré, Paris, 1909, 200.

16 Bang, I., Der Blutzucker, Wiesbaden, 1913, 74.

took place. Using animals with ligated ureters, Weyert[17] found that after the injection of 4.4 to 5.8 gm. of dextrose per kilo, the blood sugar was down to nearly normal limits in 3 hours, and Harley[18] found that with a dosage of 10 gm. per kilo, the blood sugar fell to normal in from 3 to 6 hours.

Weyert[17] records that the sugar concentration of the lymph runs close to that of the blood. He also found traces of sugar in the cerebrospinal fluid and in the vitreous humor of the eye. None can be found in the saliva except after very large doses,[9, 17, 19] nor is an appreciable amount secreted by the intestine[9, 20, 21] or by the wall of the urinary bladder.[22]

The possibility of the conversion of injected dextrose into glycogen must, of course, be considered. Voit[23] injected subcutaneously 50 gm. of dextrose into three rabbits and found in the liver only 1.4, 2.2, and 7.0 gm. of glycogen, respectively, showing that sugar is not as readily converted into glycogen by the liver when injected parenterally as when fed. Harley[18] after injecting 10 gm. of dextrose per kilo intravenously into dogs with the ureters tied, found some slight evidence of glycogen formation. Freund and Popper[24] analyzed a lobe of liver before injecting dextrose intravenously and the rest of the liver afterwards, and found a small increase in liver glycogen, which was greater if the animals had previously been starved for a short time. With starved dogs 4 gm. per kilo gave no increase in glycogen, but 7 to 11 gm. per kilo resulted in the formation of 1.3 to 7 gm. of glycogen.

Inasmuch as the injected sugar is accounted for only in small part by glycogen and by the various secretions, the blood at the same time rapidly assuming its normal content of sugar, the question naturally arises: What is the fate of the rest of the sugar? In seeking to answer this question, various facts have been brought out. Von Brasol[5] injected 12 to 18 gm. of dextrose per kilo intravenously into rabbits in 30 to 45 minutes and then analyzed the blood, the urine, and mixed samples of muscle, kidney, and liver. Estimating the relation of blood to body weight even as high as 12 per cent and even assuming that all the tissues, including bones, hair, etc., contained the same proportion of dextrose as the tissues analyzed, he calculated that from 17.5 to 28.7 per cent of the sugar administered, was still to be accounted for. Von Brasol did not determine glycogen or other substances; in fact he suggests that the missing fraction has been converted into glycogen, or lactic acid or some other substance. Butte[12] made muscle analyses after injecting intravenously 4 gm. of dextrose per kilo into a dog. Assuming

[17] Weyert, F., *Arch. f. Physiol.*, 1891, 187.

[18] Harley, V., *Arch. f. Physiol.*, 1893, Supplement, 46.

[19] Jappelli, A., *Ztschr. f. Biol.*, 1908, li, 435.

[20] Bang, I., Der Blutzucker, Wiesbaden, 1913, 80.

[21] Kleiner, I. S., *Jour. Exper. Med.*, 1911, xiv, 274.

[22] Kleiner, I. S., *Jour. Exper. Med.*, 1913, xviii, 310.

[23] Voit, C., *Ztschr. f. Biol.*, 1891, xxviii, 245.

[24] Freund, E., and Popper, H., *Biochem. Ztschr.*, 1912, xli, 56.

that the muscle contained no sugar before the injection, he noted a rise to 0.42 per cent half an hour after the injection and then a progressive fall in the percentage of dextrose. This demonstrates, according to Butte, that one part of the injected sugar is transformed in certain organs, although he does not mention the likelihood of bacterial action in a dead animal. In two experiments on rabbits Bang[20] analyzed the liver, skin, blood and lymph, kidneys and urine, muscles, intestines, and bones for free dextrose after injecting 4 gm. intravenously into each animal. The total amount recovered was 47.9 and 75.5 per cent, respectively. But, according to Bang, even these figures are too high because (a) preformed sugar was not determined, (b) the tissues were not blood-free and hence blood sugar was counted twice, and (c) all reduction was not necessarily due to sugar.

The rapid transformation of dextrose into simpler substances, particularly lactic acid, has been suggested by many investigators. Apparently the only one who actually tested for lactic acid after intravenous injections of dextrose was Harley.[18] He found a higher percentage of lactic acid in the blood after dextrose injections into animals with the ureters tied, and also an increase in this substance in the liver and muscle. He also obtained qualitative tests for ethyl alcohol and acetone in the blood. Although some workers have brought forth evidence in favor of a protein origin for lactic acid, there seems to be no doubt that it can be formed from sugar. For example, Embden and his collaborators[25] by perfusing livers with blood found that the liver could form lactic acid from glycogen in the liver or dextrose in the blood. More recently Levene and Meyer[26] have observed a direct conversion of sugar into lactic acid by leukocytes.

An increase in the respiratory quotient after intravenous dextrose injections has been observed by Harley,[27] Verzár and Fejér,[28] and others.

The following points appear, therefore, to be established: (1) The intravenous injection of large quantities of dextrose is followed by an increase in the percentage of sugar in the blood. (2) The high content of sugar in the blood falls rapidly after the end of the injection and assumes the normal value in a comparatively short time, even when the ureters are tied. (3) The kidneys eliminate a considerable, but variable, fraction of the injected sugar. (4) No sugar, or mere traces are to be found in the cerebrospinal fluid, saliva, intestinal secretions, or in the secretion of the mucosa of the urinary bladder. (5) An increase of glycogen in the liver occurs with very large doses, but it accounts for only a small fraction of the sugar injected. (6) Tissue analyses, although few in number, show that some of the sugar is to be found unaltered in various organs. (7) Some of the sugar is oxidized, or at least the respiratory quotient is increased.

---

[25] Embden, G., *Centralbl. f. Physiol.*, 1904–05, xviii, 832. Embden, G., and Kraus, F., *Biochem. Ztschr.*, 1912, xlv, 1.

[26] Levene, P. A., and Meyer, G. M., *Jour. Biol. Chem.*, 1912, xi, 361.

[27] Harley, V., *Jour. Physiol.*, 1894, xv, 139.

[28] Verzár, F., and von Fejér, A., *Biochem. Ztschr.*, 1913, liii, 146.

The chief purpose of the present work was to study the disappearance of sugar from the blood under various conditions. In order to obtain a basis of comparison a series of experiments was first carried out in which dextrose was injected into normal animals.

EXPERIMENTAL PART.[29]

The experiments were performed on dogs. Ether was administered by intratracheal insufflation, and cannulas were inserted in the left jugular vein, the right carotid artery, and the neck of the urinary bladder. In a few of the later experiments only morphine and cocaine were employed as anesthetics. In the experiments in which the kidneys were ligated, they were reached through the lumbar region. At the completion of all operative work, the animal was given morphine, usually 0.01 gm. per kilo, subcutaneously, and then the insufflation and ether anesthesia were discontinued. After $1\frac{1}{2}$ to $2\frac{1}{4}$ hours a sample of blood was taken from the artery, and a warm solution of 20 per cent dextrose was injected slowly into the jugular vein. The rate of injection was about 2.5 to 3 cc. per minute and the dosage was 4 gm. per kilo of body weight. At the middle and end of the injection and at intervals thereafter, samples of blood (from 10 to 30 gm.) were taken for analysis. Urine elimination was divided into periods corresponding to the blood samples taken. During the experiment the animal was kept on an electric thermal pad and at the end of the experiment it was chloroformed.

The blood was analyzed for dextrose by removing the protein by Reid's method,[30] and the reduction was determined by means of a Pavy[31] solution. The urine

[29] A preliminary report of this work was published by Kleiner, I. S., and Meltzer, S. J., *Am. Jour. Physiol.*, 1914, xxxiii, p. xvii.

[30] Reid, E. W., *Jour. Physiol.*, 1896, xx, 316. Vosburgh, C. H., and Richards, A. N., *Am. Jour. Physiol.*, 1903, ix, 35. Macleod, J. J. R., *Jour. Biol. Chem.*, 1908–09, v, 443.

[31] The reagent used was modified from time to time. Vernon (*Jour. Physiol.* 1902, xxviii, 156) suggested the use of twice as much Rochelle salt, potassium hydroxide, and ammonium hydroxide as Pavy originally employed. As this results in the formation of a heavy crystalline deposit in the reagent on standing, we have decreased the amount of Rochelle salt and potassium hydroxide. The resulting solution, which deposits very little precipitate, gives good results. The composition of the solution, as we now prepare it, is:

$CuSO_4$	4.158 gm.
Rochelle salt	14 "
KOH	17 "
NH_4OH (sp. gr. 0.88)	600 cc.
H_2O to	1,000 "

10 cc. of this solution corresponds to 0.005 gm. dextrose, but one should determine the exact equivalent every time the reagent is made up, and this should be checked at intervals.

was also analyzed by the Pavy method. Although this method is not as accurate as some of the other sugar methods, its rapidity, and the fact that a number of determinations can be made from a small quantity of a given solution (as each test requires only 5 mg. of dextrose), led us to use it in this investigation. In some experiments, however, the Bertrand method was used to determine the reducing power of the blood after removal of the proteins, and in a few of the experiments we have used the blood sugar method of Lewis and Benedict as modified by Myers and Bailey,[32] which we have recently adopted for use in this laboratory. With this method only 2 cc. of blood is required.

Normal Animals.

The first dextrose injections were given to seven normal animals. The following is a typical protocol.

Experiment LD 5.—White bull terrier, female; weight 6.5 kilos.

11.00. Etherized. Intratracheal insufflation begun and ether anesthesia continued by this method. Cannulas now inserted in the left jugular vein, left carotid artery, and in the neck of the bladder.

11.51. Urine obtained from bladder; 1.69 per cent dextrose.

11.56. Ether discontinued.

12.02. 6.5 cc. 1 per cent morphine sulphate injected subcutaneously.

12.13. Insufflation discontinued.

2.07. Blood taken, 22.3 gm.; 0.28 per cent dextrose.

2.09. Urine, 77 cc. + (about 5 cc. lost), 8.84 per cent = 6.81 gm. dextrose.

2.10. Injection of warm 20 per cent dextrose solution into jugular vein started.

2.34. Blood taken, 9.5 gm., 0.68 per cent (67 cc. of dextrose have been injected).

2.36. Urine, 40 cc., 6.84 per cent = 2.74 gm. (70 cc. of dextrose have been injected).

2.56. Dextrose injection ends. Total amount injected 130 cc., or 26 gm. in 46 minutes.

2.57. Blood taken, 10.0 gm., 0.93 per cent.

2.58. Urine, 81 cc., 5.50 per cent = 4.46 gm.

3.27. Blood taken, 16.6 gm., 0.40 per cent.

3.28. Urine, 51 cc., 7.32 per cent = 3.73 gm.

3.57. Blood taken, 17.9 gm., 0.30 per cent.

3.58. Urine, 11.5 cc., 10.6 per cent = 1.22 gm.

4.27. Blood taken, 21.3 gm., 0.28 per cent.

4.29. Urine, 3.2 cc., 8.59 per cent = 0.28 gm.

---

[32] Lewis, R. C., and Benedict, S. R., *Jour. Biol. Chem.*, 1915, xx, 61. Myers, V. C., and Bailey, C. V., *Post-Graduate*, 1915, xxx, 31; *Jour. Biol. Chem.*, 1916, xxiv, 147.

Summary.—$1\frac{1}{2}$ hours after the end of the dextrose injection the blood sugar fell to its original level of 0.28 per cent.

	gm.
Dextrose injected	26.0
Excess dextrose (above original value) in circulating blood at end of experiment	0.00
Dextrose contained in blood samples taken for analysis	0.13
Dextrose in urine	12.43
Dextrose accounted for	12.56
(or 48.5 per cent of the amount injected)	
Dextrose not accounted for	13.44
(or 51.5 per cent of the amount injected)	

The results of the seven experiments are given in Table I.

Considering first the glycemia, it is seen that in five out of seven experiments, the original blood sugar was high. This was to be expected, as the animals had been subjected to being tied down, to anesthesia (ether and morphine), and to operative procedure, all of which are known to raise the blood sugar.[33] An initial rapid rise in the blood sugar during the injection is indicated in each case by the figures for the beginning and middle of the injection. Then a more gradual rise occurred until the end of the injection, after which the percentage of blood sugar fell rapidly for half an hour and then more slowly. At the end of $1\frac{1}{2}$ hours the percentage of sugar in the blood fell to its original level in three experiments (Experiments 3, 5, and 46) and nearly to its original level in two others (Experiments 6 and 7). In the two remaining experiments (4 and 47) the last figures were twice as great as the first. However, in one of these two (Experiment 47) the last percentage found was, nevertheless, a low figure when compared with the rest of the series. In other words, in only one of the seven experiments did the blood sugar fail to fall to what might be termed a low figure for this series.

In Experiments LD 4, 5, and 6, and LP 46, the urine secreted during the injection contained more sugar than that secreted during the 90 minute after-period. This was due to a greater volume of urine being secreted at first rather than a higher percentage of sugar.

[33] For a discussion of these points see Shaffer, P. A., *Jour. Biol. Chem.*, 1914, xix, 297. Loewy, A., and Rosenberg, S., *Biochem. Ztschr.*, 1913, lvi, 114. Hirsch, E., and Reinbach, H., *Ztschr. f. physiol. Chem.*, 1914, xci, 292.

TABLE I.

Intravenous Injection of Dextrose into Normal Dogs.

No. of experiment.	Dextrose injected (4 gm. per kilo).		Blood sugar before injection.	Middle of injection				End of injection.				½ hr. after injection.				1 hr. after injection.				1½ hrs. after injection				Total volume of urine.	Total amount of sugar in urine.		Sugar not accounted for by blood and urine. Per cent of total amount injected.
				Blood sugar.	Urine.			Blood sugar.	Urine.			Blood sugar.	Urine.			Blood sugar.	Urine.			Blood sugar.	Urine.					Per cent of amount injected.	
					Volume.	Dextrose.			Volume.	Dextrose.			Volume.	Dextrose.			Volume.	Dextrose.			Volume.	Dextrose.					
	gm.	cc.	per cent	per cent	cc.	per cent	gm.	per cent	cc.	per cent	gm.	per cent	cc.	per cent	gm.	per cent	cc.	per cent	gm.	per cent	cc.	per cent	gm.	cc.	gm.	per cent	per cent
LD3...	29.6	148	0.23	0.85	0			0.98	0			0.63	10.5	3.68	0.39	0.33	1.0	5.5	0.05	0.24	2.7	3.7	0.10	15	0.54	1.8	97.0
LD4...	26.8	134	0.20	0.71	35	6.62	2.32	0.80	81	7.31	5.92	0.45	63	7.70	4.86	0.47	8.3	9.38	0.78	0.41	9.9	9.06	0.90	197	14.78	55.1	39.9
LD5...	26.0	130	0.28	0.68	40	6.84	2.74	0.93	81	5.50	4.46	0.40	51	7.32	3.73	0.30	11.5	10.6	1.22	0.28	3.2	8.59	0.28	187	12.43	47.8	51.5
LD6...	26.0	130	0.19	0.56	32	9.80	3.14	0.72	61	9.80	5.98	0.40	49	9.22	4.52	0.28	13.5	10.9	1.47	0.25	11.5	11.54	1.33	167	16.44	63.2	34.2
LD7...	30.0	150	0.11	0.49				0.50				0.25	121	8.68	10.50	0.18				0.15	50	8.06	4.03	171	14.53	48.4	50.4
LP46*..	34.0	170	0.18					0.70	209	7.42	15.51									0.20	112	10.53	11.79	321	27.30	80.3	19.4
LP47*..	35.6	178	0.11					0.76	112	10.10	11.31									0.25	92	13.32	12.25	204	23.56	66.2	31.4
Average			0.19	0.16†				0.77				0.43†				0.33†				0.25						60.2‡ 51.8§	37.8‡ 46.3§

* Only morphine anesthesia. † Average of five. ‡ Average of six. § Average of seven.

Indeed, the urine of the after-period was usually of a higher concentration, and in Experiment LP 47 this was sufficient to cause a greater excretion of sugar in the after-period than in the injection period.

The small flow of urine in Experiment LD 3 was probably due to over-etherization. Excluding this experiment, the average amount found in the urine was 60.2 per cent of the sugar injected. If we now estimate the excess sugar still circulating in the blood (calculating the blood equal to 7 per cent of the body weight) and add this blood sugar to the urinary sugar, we have an average of 62.2 per cent of the injected amount, leaving 37.8 per cent not accounted for. If we include Experiment LD 3, this average is raised to 46.3 per cent not accounted for.

It thus appears from our own experiments on normal animals that approximately 60.2 per cent of the sugar was excreted by the kidneys, while of the remaining 39.8 per cent only an insignificant fraction (2 per cent on an average) remained in the circulation.

Nephrectomized Animals.

In one of our experiments on normal animals (Experiment LD 3) there occurred, as mentioned above, an almost complete suppression of the kidney function during and after the intravenous infusion of dextrose. Nevertheless, the blood sugar returned to its original value as quickly as in the other experiments. We now performed some experiments upon animals after ligating or removing the kidneys. The results of five experiments are summarized in Table II.

The following is a typical protocol.

Experiment LD 33.—Fox-terrier, male; weight 6.75 kilos.

10.18. Etherized. Intratracheal insufflation begun and ether anesthesia continued by this method. The kidneys were now exposed and ligated and cannulas inserted in left jugular vein and right carotid artery.

11.15. Ether discontinued.

11.26. Lid reflex present.

11.27. Blood taken, 11.6 gm., 0.28 per cent.

11.31. Insufflation discontinued.

11.33. 1 cc. 1 per cent morphine sulphate injected intramuscularly.

12.27. Blood taken, 11.35 gm., 0.27 per cent.

12.28. Injection of warm 20 per cent dextrose solution into jugular vein started.

1.22. Dextrose injection ended. Total amount introduced, 135 cc. or 27 gm. in 54 minutes.

1.23. Blood taken, 11.9 gm., 1.08 per cent.

1.29. 0.5 cc. 1 per cent morphine sulphate injected intramuscularly.

1.54. Blood taken, 11.5 gm., 0.69 per cent.

2.24. Blood taken, 14.9 gm., 0.40 per cent.

2.54. Blood taken, 15.95 gm., 0.32 per cent.

Summary.—1½ hours after the end of the injection the blood sugar fell to 0.32 per cent; *i.e.*, only 0.05 above its original level of 0.27 per cent.

		gm.
Dextrose injected		27.0
Excess dextrose (above original value) in circulating blood at end of experiment	about	0.27
Dextrose contained in blood samples taken for analysis	about	0.17
Total dextrose thus accounted for (or 1.7 per cent of the amount injected)		0.44
Dextrose not accounted for (or 98.3 per cent of the amount injected)		26.56

TABLE II.

Intravenous Injection of Dextrose into Nephrectomized Dogs.

No. of experiment.	Dextrose injected (4 gm. per kilo).		Blood sugar before injection.		Blood sugar at end of injection.	Blood sugar ½ hr. after injection.	Blood sugar 1 hr. after injection.	Blood sugar 1½ hrs. after injection.	Blood sugar 2 hrs. after injection.	Sugar not accounted for. Per cent of total amount injected.
			Before morphine.	After morphine.						
	gm.	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
LD 32	11.0	55		0.47	1.09	0.93	0.70		0.45	100
LD 33	27.0	135	0.28	0.27	1.08	0.69	0.40	0.32		98.3
LD 34	42.0	210	0.30	0.35	1.0		0.55	0.46	0.29	99.6
LP 48*	43.4	217		0.10	0.62			0.13		99.5
LP 49*	37.0	185		0.28	1.03			0.27		100
Average				0.29	0.96			0.295		99.5

* In these two experiments the kidneys were exposed and prepared for ligation under ether anesthesia, but they were not actually ligated until the effects of the ether had worn off.

In these animals the blood sugar rises to a higher level, but this is not as great as might be expected. In the normal dogs the average figures were 0.19 before injection and 0.77 at the end, a rise of 0.58 per cent; in the nephrectomized animals the corresponding figures are 0.29 and 0.96, a rise of 0.67 per cent; hence there is a difference between the two series of only 0.09 per cent. If we now estimate from this figure how much more sugar there is circulating in the blood in this series, we find that it amounts to less than 1 gm. Therefore during the injection the organism without the kidney has been enabled to rid its blood of nearly as much sugar as if it had its renal function intact. After the discontinuation of the injection the fall in the glycemia is most rapid during the first hour and then becomes slower. In Experiments LD 33, LD 34, LP 48, and LP 49 the blood sugar is either at its original level or only slightly higher at the end of $1\frac{1}{2}$ hours, and in Experiments LD 32 and 34 the figures for 2 hours show that by this time the blood sugar has fallen to or below this value. In Experiment 32 no sample was taken at the end of $1\frac{1}{2}$ hours, but the average for the other four experiments for this time was 0.30 per cent, while the average before injection was 0.25 per cent in the same four.

It is thus evident that after an intravenous injection of dextrose the blood sugar does not rise much higher in nephrectomized animals than in normal animals, and after injection gradually falls to or nearly to its original level. This indicates, of course, that practically all the injected sugar has left the blood stream; and that therefore the body is able to dispose of large amounts of injected dextrose even without the assistance of the kidneys. Furthermore, the fact that this was accomplished in the same length of time as in the normal series, the dosage and other experimental conditions remaining the same, shows that the presence or absence of the kidney has very little influence on the rate of disappearance of dextrose from the blood.

The obvious explanation which offers itself at first thought is that the greater part of the injected sugar is rapidly converted into glycogen by the liver. We have performed some experiments which were designed to exclude this possibility. Dextrose was injected into dogs in which there was practically no circulation posterior to the diaphragm. Animals can easily be prepared in this manner since, with

the aid of intratracheal insufflation, the thorax can be opened and the aorta and vena cava inferior tied near the diaphragm with great facility.

Animals with a Circulation only Anterior to the Diaphragm.

The animals were prepared as follows: An opening was made in the left side of the thorax (under intratracheal insufflation anesthesia) and the aorta and vena cava were ligated as near the diaphragm as possible. The aorta was ligated first, and before ligating the vena cava, pressure was applied to the abdomen in order to increase the amount of blood in the thorax. In some cases Ringer's solution or saline, in others adrenalin, or both, were injected to maintain the blood pressure. These animals, then, had no abdominal circulation; the liver could store no glycogen; the kidneys, pancreas, and adrenals, could not function. Into such animals, we injected dextrose as before and estimated the glycemia at intervals. Some experiments were complicated by such variations as removal of the thyroids, tying the thoracic duct, etc. These additional factors exerted no appreciable influence on the results, and we shall not deal with them in particular. Some of the experiments are summarized in Table III.

A typical experiment of this series is the following:

Experiment LD 30.—Dog; weight 7.5 kilos.

1.40. Etherized. Intratracheal insufflation begun and ether anesthesia continued by this method. Cannulas now inserted in left jugular vein and right carotid artery. Thoracic duct exposed. Opening made in left side of thorax and aorta tied near diaphragm. After pressure on the abdomen the vena cava inferior was tied near diaphragm.

2.43. Wound in thorax closed.

2.44. Ether discontinued.

2.46. Thoracic duct tied.

2.47. Blood taken, 10.9 gm., 0.07 per cent.

2.48–2.50. 40 cc. warm sterile Ringer's solution injected in left jugular vein.

3.04–3.05. 20 cc. Ringer's solution injected.

3.18. 10 cc. Ringer's solution injected.

3.23. Pulse poor. Dog restless. 20 cc. Ringer's solution injected.

3.31–3.32. 20 cc. Ringer's solution injected. (Total quantity Ringer's solution injected, 110 cc. during 44 minutes).

3.40. 1 cc. 1 per cent morphine injected intramuscularly in left fore-leg.

3.49. Blood taken, 10.8 gm., 0.05 per cent.

3.50. Injection of warm 20 per cent dextrose in left jugular vein started.

4.30. Dextrose injection ended. Total amount injected, 150 cc. 20 per cent dextrose or 30 gm., in 40 minutes.

4.32. Blood taken, 14.7 gm., 1.65 per cent.
4.48. Pulse good.
5.27. Blood taken, 22.6 gm., 1.16 per cent.

Summary.—Estimated weight of anterior part of animal; *i.e.*, one half of total body weight.......................... 3,750 gm.

Quantity of blood circulating (7 per cent of weight)......	262	cc.
Fluid injected....................................	260	"
	522	"
Blood samples taken for analysis..............about	39	"
Total circulating fluid..........................about	483	"
Excess dextrose circulating ((1.16–0.05 per cent) × 483 cc.)	5.36	gm.
Dextrose in blood samples taken........................	0.26	"
Total dextrose accounted for......................about	5.62	"
Dextrose injected....................................	30	"

Amount of dextrose not accounted for, 24.4 gm. or 81 per cent of the quantity injected.

The interpretation of these figures is made difficult by several complications. As we injected the same amount of dextrose (4 gm. per kilo) as in the first two series, the dosage of dextrose per kilo was manifestly higher in these experiments since the posterior part of the body was not fed by the circulation. Exactly how much higher cannot be said definitely, but probably the remaining circulation received about twice as much dextrose per kilo as in the experiments on the entire animal. The infusion of Ringer's solution and the occasional use of adrenalin also make these experiments not entirely comparable with the others. However, the first blood sample was always taken after injection of Ringer's solution or adrenalin, so that a comparable initial glycemia was always ascertained.

In these experiments the blood sugar rose greatly; at the end of the injection it reached from 1.35 to 2.41 per cent, an average of 1.93 per cent for eleven experiments. In considering these figures it must be remembered that, as stated above, the dose of sugar per volume of circulating blood was about twice as high as in the other two series. In every experiment there was a rapid fall in the blood sugar after the injection had been finished. Since the samples of blood were not always taken at the same intervals, it becomes necessary to study the experiments in groups. In Experiments 55, 56, and 57 the

TABLE III.

Intravenous Injection of Dextrose into Dogs with Aorta and Vena Cava Ligated near the Diaphragm.

No. of experiment.	Dextrose injected (4 gm. per kilo).		Blood sugar before injection.		Blood sugar at end of injection.	Blood sugar ½ hr. after injection.	Blood sugar 1 hr. after injection.	Blood sugar 1½ hrs. after injection.	Blood sugar 2 hrs. after injection.	Muscle before injection.		Muscle after injection.		Sugar not accounted for. Per cent of total amount injected.	
										Dextrose.	Polysaccharides.	Dextrose.	Polysaccharides.	By blood.	By blood and muscle.
	gm.	cc.	per cent		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
LD18......	18.0	90			2.33		1.44							82	
LD20......	25.6	128	0.15	(3 hrs. later) 0.11	2.03		1.16							87	
LD21* ‡....	28.0	140	0.14	(1 hr. later) 0.04	2.34		1.55							80	
LD28* §....	41.0	205		0.19	2.18		1.58							78	
LD30* ‖....	30.0	150	0.07	(1 hr. later) 0.05	1.65		1.16							82	
LD31† ¶....	41.0	205		0.10	1.65		1.18							83	
LD35† *** ..	27.0	135		0.40	1.81		1.34		1.13					86 (1 hr.) 89(2 hrs.)	
LD45* **...	34.0	170			1.75							0.49	0.21	74	
LD55......	37.0	176		0.33	1.35	1.02				0.26	0.15	0.36	0.24	91	82
LD56*......	29.8	144		0.15	2.41	1.70				0.40	0.18	0.71	0.50	81	49
LD57*......	36.7	183.7		0.17	1.73	1.38		0.82		0.52	0.54	0.80	0.37	91	86
Average....					1.93††										
					1.83‡‡	1.37									
					2.00§§		1.34								

* Thoracic duct ligated. † Thoracic duct ligated and thyroid glands removed.

‡ 15 cc. of Ringer's solution injected between first and second blood samples.

§ 100 cc. of Ringer's solution and 1 cc. of adrenalin (in small portions) injected before taking first blood sample.

‖ 110 cc. of Ringer's solution (in small portions) injected between first and second blood samples.

¶ 75 cc. of Ringer's solution injected before taking first blood sample.

*** Ether and magnesium sulphate anesthesia. 40 cc. of Ringer's solution injected before taking first blood sample.

** 60 cc. of Ringer's solution and 0.5 cc. of adrenalin injected before dextrose injection.

†† Average of eleven. ‡‡ Average of last three. §§ Average of first seven.

TABLE IV.

Intravenous Injection of Dextrose into Whole Dead Animals.

No. of experiment.	Interval between heart stoppage and injection.	Dextrose injected (4 gm. per kilo).		Blood sugar before injection.	After injection.						Muscle before injection.		Muscle after injection.			Dextrose not accounted for. Per cent of total amount injected.	
					Time.	Blood sugar.	Time.	Blood sugar.	Time.	Blood sugar.	Dextrose.	Polysaccharides.	Time.	Dextrose.	Polysaccharides.	By blood.	By blood and muscle.
	min.	*gm.*	*cc.*	*per cent*	*min.*	*per cent*	*min.*	*per cent*	*min.*	*per cent*	*per cent*	*per cent*	*min.*	*per cent*	*per cent*	*per cent*	*per cent*
LD48.....	3	21	105		9	1.95										56	
LD49.....	21	30	150		30	3.26	73	3.85†			0.54	0.15	83	0.88	0.83	30	0
					46	2.83*											
LD51.....	25	22	110		30	1.54					0.29	0.14	42	0.60	0.21	65	27
LD53.....	14	18	90	0.02 (?)	37	1.67	67	1.48	157	1.30(1.01)‡	0.33	0.21	161	0.45	0.22	71	62
LD54.....	10	32	160	0.23	30	2.18	60	2.03	105	1.93	0.38	0.30	126	0.64	0.60	64	6

* This sample of blood was obtained from the aorta after tying the inferior and superior venæ cavæ.

† This sample of blood was obtained from the portal circulation.

‡ 100 cc. saline were injected in order to get this sample and the figure 1.30 per cent was calculated on the assumption that the saline and blood were intimately mixed.

average percentage of sugar in the blood at the end of the injection was 1.83 per cent, and a half hour later the average was 1.37 per cent—a fall of 0.46 per cent in half an hour. In Experiments 18, 20, 21, 28, 30, 31, and 35, the blood sugar fell from an average of 2.00 per cent at the end of the injection to 1.34 per cent in an hour; in Experiment 57 the fall continued steadily from 1.73 to 1.38 in half an hour, and to 0.82 per cent in $1\frac{1}{2}$ hours. In Experiment 35 a drop in the blood sugar from 1.81 to 1.34 per cent occurred in the first hour after the end of the injection, while during the second hour only a relatively slight decrease took place; namely, to 1.13 per cent.

In every experiment a large percentage of the injected sugar was not accounted for by the sugar present in the circulating blood, from 74 to 91 per cent disappearing from the blood stream during the time of observation. Another way of demonstrating this is by estimating the percentage of sugar which would have been present in the blood if all the injected sugar was still there and comparing this figure with the actual findings, taking into account the volume of blood drawn and of fluid injected. Such estimations indicate that the blood sugar content would be about 7.7 to 7.9 per cent if all the sugar was in the circulating blood. As a matter of fact the highest blood sugar values in all these experiments ranged from 1.35 to 2.41 per cent, or an average of only 1.93 per cent. It is thus evident that the greater part of the injected sugar left the blood rapidly, in fact before the end of the injection. This disappearance of dextrose from the blood stream went on, then, entirely independently of the abdominal viscera. Hence neither the glycogenic activity of the liver nor an excessive consumption of sugar by or in any abdominal organ can account for the greater part of the dextrose which so rapidly leaves the blood after intravenous infusion.

Analysis of Muscle for Carbohydrates.—In a few of these experiments we analyzed skeletal muscles to determine whether sugar had been stored in them. Higher carbohydrates, as well as dextrose were estimated. A few preliminary studies convinced us that the carbohydrate content of normal muscle is not constant. Therefore it was necessary to determine in each experiment the amount of dextrose and higher carbohydrate present in the muscles before and after the injection.

In Experiments 55, 56, and 57 the procedure was as follows: One fore-leg was amputated before the injection of dextrose and immediately placed in the refrigerator. At the end of the experiment the animal was killed, the other fore-leg removed and also placed in the refrigerator. The muscles were dissected off, put through the grinder twice, and extracted with water in the presence of toluene for about 18 hours in the refrigerator. The fluids were then pressed out and the residue again extracted with water for about 2 hours. All fluids obtained were immediately boiled after adding a little acetic acid and filtered through glass wool. The residue, pressing cloth, etc., were thoroughly extracted with boiling water. The combined fluids were then treated with phosphotungstic acid solution while boiling, filtered, neutralized with sodium hydroxide, acidified with acetic acid, and finally concentrated to a definite volume on the water bath. Estimations of the reducing power were made before and after hydrolysis and the results expressed as dextrose. Hydrolysis was effected by boiling under a reflux condenser for $1\frac{1}{2}$ hours in the presence of 1.8 per cent hydrochloric acid.

In all three experiments there was an increase in the total carbohydrates (*i.e.*, the amount found after hydrolysis) after the injection. There was, however, some slight difference with regard to the behavior of the two types of carbohydrates. In Experiments 55 and 56 the increase in dextrose is practically the same as that of the higher carbohydrates; namely, 0.10 per cent for dextrose and 0.09 per cent for higher carbohydrates in Experiment 55; and 0.31 per cent for dextrose and 0.32 per cent for higher carbohydrates in Experiment 56. In both experiments the time was half an hour. In Experiment 57, where the time was $1\frac{1}{2}$ hours, there was an increase of 0.28 per cent in the dextrose and a decrease of 0.17 per cent in the higher carbohydrates. The fact that the higher carbohydrates were found to be increased after 30 minutes and decreased after 90 minutes might indicate that the higher carbohydrates were more readily utilized than the lower ones, or that dextrose before being oxidized by the muscle must be converted into a polysaccharide. Our evidence, however, comes from only three experiments, and consequently we cannot attach much importance to it.

Does this increase in the carbohydrate content of muscle account for all the injected dextrose which disappeared from the circulation? Consideration of the following figures will show that we are not entitled to answer this in the affirmative. If we assume that the muscle tissue of the dog constitutes approximately 43 per cent of

the body weight, as in man,[34] and that half the body is reached by the circulating blood, we can estimate roughly the increase in carbohydrates of the muscles. Such calculations show only comparatively small percentages of the injected dextrose. Thus in Experiment LD 55 the amount not accounted for is reduced from 91 to 82 per cent by taking the muscle carbohydrates into consideration; in Experiment LD 57 the reduction is from 91 to 86 per cent; and in Experiment LD 56 it is somewhat greater, namely, from 81 to 49 per cent. From this it seems evident that the anterior muscles in these experiments did not contain all the missing sugar. On the other hand, it must be admitted that one cannot draw definite conclusions from the muscle analyses, for which great accuracy cannot be claimed. Furthermore it is possible that, in these "anterior" animals, loss of dextrose may occur through the tissue spaces into the posterior part of the body, which we have not included in our estimations. Finally we have not examined other tissues, *e.g.*, the brain, lungs, spinal fluid, bone marrow, etc., where the carbohydrates might also have increased in amount.

Thus, while we cannot claim that the experiments on the muscles in anterior animals throw definite light upon the fate of all the dextrose which disappears from the circulation after an intravenous injection, the fact remains that a notable increase in the carbohydrate content of the muscles was found.

Experiments with Intravenous Injections of Dextrose into Whole Dead Animals.

The foregoing experiments brought up the question whether this passage of sugar from the blood into the tissues is a vital process; this led to a series of experiments in which dextrose was injected into dead animals.

Method.—The dogs were prepared as in the experiments in Tables I and III on normal and anterior animals, except that, under light chloroform insufflation anesthesia, three or four ribs were resected on the left side in order to expose the

[34] Vierordt, H., Anatomische physiologische und physikalische Daten und Tabellen, 3rd edition, Jena, 1906, 44.

heart. Then full chloroform vapor was given until the heart stopped. In some cases one fore-leg was then removed for analysis, all bleeding points being carefully ligated. Continuing the artificial respiration and massaging the heart by hand, we injected the dextrose solution intravenously, much more quickly, however, than in the experiments upon living animals. The rhythmic heart massage was continued for 5 or 10 minutes, and blood samples were taken from the carotid while massaging the heart.

The following is a protocol of one experiment.

Experiment LD 54.—Dog, male; weight 8 kilos.

10.35. Etherized. Intratracheal insufflation begun and chloroform anesthesia produced by this method. Cannulas were inserted in the left jugular vein and right carotid artery. The thorax was opened on the left side, four ribs being resected and the heart exposed. The left fore-leg was then amputated.

12.08. Left fore-leg placed in refrigerator.

12.10. Full chloroform vapor given.

12.20. The fibrillary twitchings of the heart have ceased.

12.23–12.24. Blood taken from the carotid artery, 23.03 gm., 0.23 per cent.

12.30–12.32½. 160 cc. of warm 20 per cent dextrose solution injected into left jugular vein, followed by 3 cc. 0.9 per cent sodium chloride. Heart massaged during the injection.

12.32½–12.36. Massaging of heart continued.

1.02–1.04. Blood taken from carotid, 20.04 gm., 2.18 per cent.

1.31–1.34. Blood taken from carotid, 16.07 gm., 2.03 per cent.

2.17–2.18. Blood taken from carotid, 20.74 gm., 1.93 per cent.

2.39. Right fore-leg removed and put in refrigerator.

Analysis of Muscle of Fore-Legs.

Control muscle: 0.38 per cent before hydrolysis (dextrose).
0.68 per cent after hydrolysis (total carbohydrates),
or 0.30 per cent polysaccharides.

Muscle after dextrose injection: 0.64 per cent before hydrolysis (dextrose).
1.24 per cent after hydrolysis (total carbohydrates),
or 0.60 per cent polysaccharides.

Increase in total carbohydrates, 0.56 per cent.

Summary.—Amount of dextrose injected, 32 gm. 1¾ hours after the end of the injection, the blood sugar was 1.93, or 1.70 per cent above its original value. There was estimated to be about 636 cc. of blood in the blood vessels at the time of taking the last blood sample.

Increase in dextrose in blood (636 $\times$ 1.70 per cent) ....10.8 gm.
Dextrose removed in blood samples.................. 0.7 "
Total excess dextrose in blood.......................11.5 "
or 36 per cent of amount injected.

Therefore 64 per cent not accounted for by blood.

The amount of muscle was estimated to be 3,300 gm.; that is, 43 per cent of (8,000 gm. (body weight)–340 gm. (amputated leg)).

Increase in total carbohydrates in muscle (3,300 $\times$ 0.56 per cent)	18.5 gm.
Total excess dextrose in blood	11.5 "
Total dextrose accounted for by blood and muscle	30.0 "

or 94 per cent of the amount injected.

Therefore, 6 per cent not accounted for.

From Table IV, which summarizes the experiments upon whole dead animals, it is seen that the blood sugar content never reached 4.4 to 4.75 per cent, which we estimate would have been the percentage if all the sugar injected had remained in the blood. The highest blood sugar percentage found was 3.85 per cent in the portal blood in Experiment LD 49; the lowest was 1.48 per cent (except the 1.30 per cent of the same experiment, which is not absolutely reliable because of the method used in obtaining this blood sample). The irregularity in the figures of Experiment LD 49 is due to the fact that the various samples were taken from different parts of the circulation. Although one cannot compute the total amount of sugar in the blood in the dead animal experiments with accuracy, it seemed desirable to obtain a basis of comparison with the other series. We therefore give the figures in the column, "Dextrose not accounted for by blood," estimating the blood at 7 per cent of the body weight and using the last blood sugar figure obtained (except in Experiment LD 49 in which the average was used). It is thus seen that from 30 to 71 per cent of the sugar injected was not present in the blood.

In four of these experiments muscle taken before and after the injections was analyzed, and the increase in total carbohydrates indicated that a considerable proportion of injected sugar was to be found in that tissue. In Experiment LD 49 the dextrose content of the muscle rose from 0.54 to 0.88 per cent in 83 minutes, and the higher carbohydrates from 0.15 to 0.83 per cent. This increase in total carbohydrates of the muscle[35] accounted for practically the entire 30 per cent of the injected sugar which was still unaccounted for by the blood. A similar result occurred in Experiment LD 54,

[35] The proportion of muscle in the body was assumed to be 43 per cent of the body weight.

as all but 6 per cent of the injected sugar was accounted for by the blood and muscle analyses together. Here the rise in muscle dextrose was from 0.38 to 0.64 per cent, and in muscle polysaccharides from 0.30 to 0.60 per cent, the time being about 2 hours. In fact this result was the most striking of all, for here the muscle contained about 58 per cent of the injected dextrose. In Experiment LD 51 the amount of sugar still unaccounted for after 42 minutes was reduced to 27 per cent by the muscle carbohydrates, the dextrose increasing from 0.29 to 0.60 per cent, while the higher sugars increased but little; namely, from 0.14 to 0.21 per cent. This was the shortest of the four experiments. In Experiment LD 53 there was very little increase in the dextrose of the muscle, from 0.33 to 0.45 per cent, and none in the polysaccharide content. As this was the longest experiment ($2\frac{2}{3}$ hours), bacterial activity might be thought of as an explanation of the finding of the smallest increase of carbohydrate in the muscle. The increase in the polysaccharide content of the muscles in three of the four experiments is possibly significant, indicating perhaps a condensation of dextrose, after death, within the muscles.

Experiments with Intravenous Injections of Dextrose into the Anterior Parts of Dead Animals.

In Table V are given the results of four experiments upon dead anterior animals. These experiments were similar to the preceding series except that, before causing the heart to stop beating, the aorta and vena cava were ligated near the diaphragm.

In these experiments we again find high blood sugar values, and again we may compare them with the values which we estimated would exist if all the injected sugar had remained within the blood vessels, making due allowance for fluid injected and for blood samples drawn. The calculations give from 7.3 to 7.9 per cent blood sugar. It is seen that the percentages of sugar are actually much lower; namely, 2.24 to 3.42 per cent, showing that a good deal of the sugar injected into these dead stumps left the circulation rapidly. From Experiments 50 and 58 it would seem that this occurred most quickly in the first 30 to 45 minutes and then proceeded more slowly.

In three of the experiments the muscles were analyzed before and after the injection, and it was found that the muscles were richer in

TABLE V.

Intravenous Injection of Dextrose into the Anterior Parts of Dead Animals.

No. of experiment.	Interval between heart stoppage and injection.	Dextrose injected (4 gm. per kilo).		Blood sugar before injection.	After injection.				Muscle before injection.		Muscle after injection.			Dextrose not accounted for. Per cent of total amount injected.	
					Time.	Blood sugar.	Time.	Blood sugar.	Dextrose.	Polysaccharides.	Time.	Dextrose.	Polysaccharides.	By blood.	By blood and muscle.
	min	*gm.*	*cc.*	*per cent*	*min.*	*per cent*	*min.*	*per cent*	*per cent*	*per cent*	*min.*	*per cent*	*per cent*	*per cent*	*per cent*
LD47*..................	2	27.0	135		15	3.27								55	
LD50*..................	42	23.0	115		18	3.42	43	3.07	0.47	0.57	128	1.03	0.57	58	30
					21	3.39	68	2.92†							
LD58..................	0	31.7	156	0.09	30	2.32	70	2.24	0.44	0.39	88	0.74	0.42	73	56
LD59..................	2	36.0	180	0.16			60	2.34	0.52	0.43	81	0.84	0.51	71	52

* Thoracic duct ligated also.

† This sample of blood was obtained from the aorta after tying the inferior and superior venæ cavæ.

dextrose after the injection than before. The percentage of muscle dextrose rose from 0.47 to 1.03, from 0.44 to 0.74, and from 0.52 to 0.84 per cent, respectively. The figures given in the last two columns of the table were obtained on the assumption that the injected sugar reached only half the body, and further that this half contained the same proportions of blood and muscle as the whole body; namely, 7 and 43 per cent, respectively. The first of these two columns shows that a large proportion of the injected sugar had disappeared from the circulation. In Experiment LD 47 about 55 per cent was estimated to have left the blood in 15 minutes. In the other three experiments, each lasting about 70 minutes, the amount of sugar lost from the blood was about 58, 71, and 73 per cent. From the last column of the table we see that even when we deduct the sugar found in the muscle tissue there is still from 30 to 56 per cent not accounted for. In none of these experiments were we able to account for practically all the injected sugar by the muscle and blood analyses, as in two of the experiments on the whole dead animals.

The increase in the higher carbohydrates of the muscle in these experiments is negligible. In Experiment LD 50 there was no increase; in Experiment LD 58 the increase was from 0.39 to 0.42 per cent; and in Experiment LD 59 from 0.43 to 0.51 per cent. These results are in contrast with those found on the entire dead animals (Table IV) in which there was a considerable increase in the muscle polysaccharides in two experiments and a small increase in another. This difference may perhaps be brought into connection with the observation made by Levene and Meyer;[36] namely, that the pulp or juices of various tissues of the dog are able to convert dextrose into a higher carbohydrate if they are activated by spleen juice. In the anterior animal such a mixture of the products of the spleen and tissues by means of an artificial circulation is excluded.

DISCUSSION.

Large amounts of dextrose were injected intravenously into normal dogs. A variable proportion (an average of about 60 per cent) was eliminated by the kidney during the injection and the 90 minutes

[36] Levene, P. A., and Meyer, G. M., *Jour. Biol. Chem.*, 1912, xi, 353.

which followed. By the end of this time the percentage of sugar in the blood had fallen to, or nearly to, the level found before the injection. Consequently about 40 per cent of the injected sugar was not accounted for by the blood and urine analyses. When the same amount of sugar was injected into nephrectomized dogs, the blood sugar, while rising somewhat higher in the course of the experiment, fell to its original level as quickly as in the normal animals. Therefore nearly all the sugar had left the blood in the same length of time without the aid of the kidneys. When dextrose was injected intravenously into animals which had practically no circulation posterior to the diaphragm, the blood sugar did not reach the height which it would have attained if all the sugar had remained in the blood vessels. Since the dosage, however, was based on the weight of the entire animal, and as these dogs had only about half their circulation, the dosage was really about twice as great as in the first two series. For this reason an exact comparison cannot be made. Nevertheless, it was seen that the blood sugar gradually fell and that from 74 to 91 per cent of the injected sugar had left the circulation during the injection and the after-period, which was usually not more than 1 hour. Even in dead animals the blood sugar did not rise to nearly the percentage it would have reached if the injected sugar had remained in the blood vessels. Here, too, the percentage gradually fell, showing that from 30 to 71 per cent had left the blood without any vital forces taking part. In the last series, the dead anterior animals, a similar result occurred; from 55 to 73 per cent of the injected sugar could not be accounted for by the blood.

All these experiments show that the injected sugar disappeared quickly from the circulation. The first question that arises is: Is all this sugar rapidly burned up by the organism? The oxidation of carbohydrate in the animal body is usually tested by determining the respiratory quotient, a rapid rise of which to or near to unity being taken as an indication of sugar combustion. This has not been done by us, but it is well known that after the ingestion or injection of dextrose into the normal animal a rise in the respiratory quotient occurs. There is, however, no reason to assume that a nephrectomized animal will burn dextrose twice as fast as a normal animal. Yet this would have to be the assumption if we were to explain the

results of our normal and nephrectomized series on the basis of a rapid combustion. Tangl,[37] indeed, has found that after nephrectomy the gas exchange is diminished for $3\frac{1}{2}$ to 4 hours, after which it begins to rise slowly. It therefore seems that a nephrectomized animal does not have a greater metabolism than a normal one during our experiments and probably does not oxidize more sugar. Another argument against rapid combustion is the disappearance of large amounts of sugar from the blood of dead animals. Here it can hardly be a question of increased metabolism.

The transformation of dextrose into glycogen in the liver is undoubtedly only a small factor in the disposal of intravenously injected glucose. This is evident from the work of Harley[18] and, more especially, of Freund and Popper.[24] The latter analyzed lobes of the liver before and after dextrose injection, and, when using a dosage like ours, found no increase in the glycogen content. Our experiments upon anterior animals, in which dextrose disappeared from the blood stream when all the abdominal viscera were cut off from the circulation, demonstrate clearly that the glycogenic function of the liver is not of great significance in tracing the fate of intravenously injected dextrose.

Undoubtedly one of the most important factors is the passage of the sugar into the surrounding soft tissues. In two experiments upon the dead entire animal practically all the dextrose not found in the blood was accounted for by the increased amount of sugar in the muscles. In the other experiments upon dead whole animals and dead anterior animals, from 9 to 38 per cent was estimated to be present in the muscles, and in three living anterior animals the estimations for muscle sugar were 5, 9, and 32 per cent of the amount injected. Evidently, a large and variable proportion of the injected dextrose simply passes through the capillaries into the muscle tissue. No doubt, the same process occurs in other parts of the body, more especially in all the soft tissues. The rapid disappearance of sugar from the blood is therefore, to a large extent at least, not difficult to explain.

However, the passage of dextrose into the tissues is not the only cause for its disappearance, for even in the dead animal experiments

[37] Tangl, F., *Biochem. Ztschr.*, 1911, xxxiv, 1.

we could not account for all the injected sugar in any of the anterior experiments or in two of the four experiments upon the dead whole animal. It is possible that some part of the dextrose is converted into higher carbohydrates in certain tissues. In fact some evidence, presented in the foregoing pages, indicates that this does occur in the muscles. In the living anterior experiments an increase in the higher carbohydrates of muscle occurred in two out of the three cases in which the muscle was analyzed. The same result was obtained in the dead whole animals in three out of four tests, while in the dead anterior animals there was little or no formation of polysaccharides. Apparently a condensation does occur in the muscles. But this does not account for any more of the sugar which has disappeared from the blood because it has already been included in our estimations. It may be that such a reaction occurs to the same or to a greater extent in other tissues and, in that way, a large part of the missing fraction would be accounted for.

The fact that in the dead whole animal a considerable condensation to a higher carbohydrate occurred in the muscles, while in the anterior ones very little was evident, suggests that this reaction may be influenced by an intra-abdominal organ. In the living anterior animals, however, a similar polymerization was found. To harmonize this with the above suggestion one would be obliged to assume that the almost insignificant amount of circulation still existing posterior to the diaphragm in living anterior animals suffices to bring from the abdomen the substance required to aid in the condensation. In the dead anterior animals the artificial circulation maintained at times by massaging the heart would not be enough to bring this about.

As other investigators have suggested, it is possible that a part of the injected dextrose is broken down to lower incompletely oxidized compounds. It would therefore not be entirely burned and at the same time, it would not be found as dextrose. We have no evidence to offer on this point.

SUMMARY.

1. As has been found by other investigators, when a large amount of dextrose is injected intravenously into a normal dog it disappears from the circulating blood in about 90 minutes after the end of the

injection. Varying amounts (an average of 60 per cent) are excreted in the urine.

2. Even in nephrectomized animals the same quantity will leave the circulation in the same length of time as in normal animals.

3. This phenomenon seems to be, at least to a great extent, independent of vital processes, since dextrose, after intravenous injection into dead animals, is found to leave the blood rapidly.

4. The phenomenon is independent of the important abdominal organs, for it also occurs in animals (living or dead) in which the aorta and inferior vena cava have been ligated near the diaphragm, thus abolishing most of the circulation posterior to the diaphragm.

5. The fact that a considerable amount of the sugar passes from the circulation into the surrounding tissues was established by finding an increase in the carbohydrates of the muscle tissue. This was done in the case of the living anterior animals and in the whole and anterior dead animals. In most of these experiments there was also evidence of the formation of polysaccharides in the muscle tissue.

THE FUNCTION OF THE KIDNEY WHEN DEPRIVED OF ITS NERVES.*

By WILLIAM C. QUINBY, M.D.

(*From the James B. Brady Urological Institute, Johns Hopkins Hospital, Baltimore.*)

Plates 81 to 83.

(Received for publication, February 14, 1916.)

The question as to what may be the function of a kidney excluded from all nervous influences is not settled at present, in spite of many experimental attempts by various observers. The reason for this lies in the fact that none of the methods employed to solve the question have been free from criticism. That the kidney is plentifully supplied with nerves is well known; but that these have any further function than that of vasomotor control has not been proved. Attempts to demonstrate secretory fibers to the kidney in either the vagus or splanchnic nerves or their branches have given only conflicting evidence.

Removal of all the nerve filaments at the renal hilus by dissection would exclude the kidney from the nervous system, so that its function could be compared with that of a normal organ. But on examining the situation more closely one finds that such method of nerve removal has been found most uncertain in its results.[1] This is due to the fact that the nerve filaments supplying the organ not only lie in a network closely applied to the renal vessels, but also run partly within the walls of these vessels. It has therefore been suggested to use some chemical means of destruction, such as painting with carbolic acid after the dissection. But even under these circumstances one is never absolutely sure that all nerves have been removed. Also, in such thin walled vessels as the renal vein, car-

* Presented in abstract at the meeting of the Federation of American Societies for Experimental Biology, Boston, Dec. 26, 1915.

[1] Cohnheim, J., and Roy, C. S., Untersuchungen über die Circulation in den Nieren, *Virchows Arch. f. Path. Anat.*, 1883, xcii, 443.

bolic acid extensively used may cause a local reaction leading to thrombosis. For these or other reasons, none of the evidence thus far brought forward in regard to the function of such kidneys is convincing.

It has been shown by workers on the methods and possibilities of blood vessel suture that a kidney removed from the body of an experimental animal, and later reimplanted by restoration of the circulation, is able to support life in a presumably normal fashion. With one exception, however, all such investigators have been engaged in demonstrating the possibility of the operation, and have not concerned themselves with the detailed physiological function of the organ.

In the present investigation it was desired to examine in detail the function of a kidney which had been removed from the body and subsequently replaced. By this method it is certain that the organ is entirely outside the sphere of all nervous influences for a time at least, if not permanently. Furthermore, the response of such a kidney to the various functional tests gives at least indirect evidence on the question of secretory innervation.

HISTORICAL.

Asher[2] is one of the most recent investigators to attempt the demonstration of secretory nerves to the kidney. With his coworkers, Pearce and Jost, he has made observations which seem to show that the vagus nerve carries secretory fibers, while the splanchnic carries inhibitory ones; at least so far as the water output of the kidney is concerned. Jungmann and Meyer,[3] after cutting the splanchnic, found an increase of urine and of sodium chloride delivered from the kidney on the same side as the severed nerve. Rhode and Ellinger,[4] however, obtained different results, which led them to believe that the splanchnic has an inhibitory action. The difficulty seems to lie in the fact that in the methods

[2] Asher, L., Die Innervation der Niere, *Deutsch. med. Wchnschr.*, 1915, xli, 1000. Asher, L., and Pearce, R. G., Die sekretorische Innervation der Niere, *Ztschr. f. Biol.*, 1914, lxiii, 83. Jost, W., Die sympathische Innervation der Niere, *Ztschr. f. Biol.*, 1914, lxiv, 441.

[3] Jungmann, P., and Meyer, E., Experimentelle Untersuchungen über die Abhängigkeit der Nierenfunktion vom Nervensystem, *Arch. f. exper. Path. u. Pharm.*, 1913, lxxiii, 49.

[4] Rhode, E., and Ellinger, P., Über die Funktion der Nierennerven, *Zentralbl. f. Physiol.*, 1913–14, xxvii, 12.

employed to elucidate the problem, the circulatory effects of vagus or splanchnic stimulation completely mask other possible effects.

More recently Pearce[5] has employed the method of Barcroft in which the oxygen consumption of the kidney is used as the index of cellular activity, and he has been unable to confirm his previous work done with Asher.

Transplantation or reimplantation of the kidney has been found surgically possible by Carrel, Stich, and a few others.[6] The most recent worker on this subject has been Lobenhoffer. His is the only work which attempts in any detailed way, to study the function of a kidney so treated. He united the severed renal vessels to those of the spleen in dogs, and was successful in ten instances. After removal of the other kidney, the water and salt output, as well as that of lactose and sugar caused by phloridzin, was studied. His results show that such a kidney is able to meet not only the ordinary demands of life, but also the excessive ones set up by the experimental injections. Further details of his work will be discussed later.

Zaaijer[7] has recently reported the survival and complete health of a dog bearing a single kidney, which had been transplanted to the iliac vessels 6 years previously.

Method.

The experiments were carried out on large dogs of both sexes. After examination of the various regions where the kidney might be placed, it was decided not to transplant the organ, but to reunite it to its own severed vessels; for in this way the nearest approach to normal physiological conditions is secured.

Although the left kidney of the dog is somewhat more accessible than the right, and has slightly longer vessels, the renal artery on the left is often bifurcated, or even may leave the aorta as two separate vessels. This reduces the caliber of the arteries and doubles the amount of time spent in sewing. The right side was chosen, therefore, in nearly all instances.

[5] Pearce, R. G., and Carter, E. P., The Influence of the Vagus Nerve on the Gaseous Metabolism of the Kidney, *Am. Jour. Physiol.*, 1915, xxxviii, 350.

[6] Carrel, A., Doppelte Nephrektomie und Reimplantation einer Niere, *Arch. f. klin. Chir.*, 1908-09, lxxxviii, 379. Stich, R., Über Gefäss- und Organtransplantation mittelst Gefässnaht, *Ergebn. d. Chir. u. Orthop.*, 1910, i, 1. Lobenhoffer, W., Funktionsprüfungen an transplantierten Nieren, *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1913, xxvi, 197.

[7] Zaaijer, J. H., Dauerresultat einer autoplastischen Nierentransplantation bei einem Hunde, *Beitr. z. klin. Chir.*, 1914, xciii, 223.

Ether was used by the intratracheal method in all the experiments. This method of administration is important, because by its use the respiratory movements can be reduced to a minimum, or even abolished. A motionless field is thus obtained which is of great aid in accurate and speedy sewing.

The animals were prepared for operation by shaving over the abdomen and far down onto the flanks. This area was then made sterile by soap and water, alcohol, ether, and tincture of iodine. A catheter was placed in the bladder, and in the case of male dogs, was carried off to the left side where it remained during the operation. The surgical asepsis was in every respect as strict as possible; gowns and gloves were worn, although during the actual suture of the blood vessels the gloves had to be removed, following which the hands were carefully coated with sterile vaselin.

Transverse Abdominal Incision.—Incision is made transversely across the abdomen just above the level of the umbilicus, and extending from the outer border of the left rectus abdominis across the right side down to the erector spinæ group of muscles (Fig. 1). This is well shown in the illustrations.[8] Failure to prolong the incision far enough towards the posterior wall of the abdominal cavity prevents easy access to the whole kidney region, and makes the suture difficult or impossible.

The abdomen is entered in layers, careful attention being paid to hemostasis. After incision of the skin and subcutaneous tissues, the fascia of the external oblique is picked up on the right, at the outer border of the rectus. It is lifted up, incised, and the incision prolonged well toward the flank. The internal oblique is similarly treated. The rectus is dissected bluntly from its underlying sheath as far as the middle line, when it is cut through. The left rectus is similarly divided. After entering the abdomen through the linea alba, the transversalis can be quickly cut down in either direction (Fig. 2). The wound gapes widely and needs no retraction. All the intestines

[8] This method of entrance to the abdominal cavity is of great aid in the performance of other operations, such as Eck fistula and those involving the biliary passages or adrenal glands. It is illustrated not because the procedure is in any way new, but because I have found that its value is not generally recognized by laboratory investigators.

except the duodenum and descending colon, are then lifted out of the abdomen to the left, where they lie covered by silk handkerchiefs impregnated with liquid vaselin (Fig. 3).

Preparation of Kidney and Renal Vessels for Suture.—After section of the peritoneum about the kidney, it is lifted out and turned toward the middle line of the body where, surrounded with gauze, it is gently held by the assistant. In this position the renal artery comes first to view, and it is carefully cleaned as far as the aorta of all surrounding tissue and nerves. This is done by blunt dissection and by wiping with dry gauze. The kidney is allowed to fall back into place, and the vein is similarly cleaned as far as the vena cava. The ureter is next stripped downward for about 6 or 7 cm. The field is then carefully washed with salt solution, every bleeding point caught and tied, and after drying, the whole is coated with liquid vaselin, including the kidney and its vessels. The two rubber-covered *serrefines* are then placed on the artery, two others on the vein, and the vessels are cut between them. The ureter is cut long. The kidney is then removed from the body and placed on a clean gauze pad. The gloves are now removed.

Vessel Suture.—The ends of the severed vessels are washed quickly with salt solution from a bulb pipette, till every trace of blood is removed. They are then coated with liquid vaselin. The adventitia is removed from the ends of the arteries in the usual way, after which the whole field within the abdomen is covered with vaselined silk handkerchiefs leaving only the stumps of the renal vessels protruding. These handkerchiefs are held in place by brass clips used by stationers, called "O.K. paper fasteners."

The most exacting and important part of the vascular suture lies in placing the three primary guide or tension stitches. This is made much easier by using a suture armed with two needles, one at either end. In this way the suture can always be passed from within the vessel outward. The circumference of the vessel ends must be accurately divided into thirds by these tension sutures.

The artery, being behind the vein, is sewed first, using an over-and-over stitch. This is followed by suture of the vein. The *serrefine* clamps are then removed from the vein and the vessel is allowed to fill under moderate pressure, rolling it a bit between the thumb

and finger. The artery is next freed while held between the thumb and finger so as partially to control the tension during the first few moments of blood flow.[9] After the vascular suture is ended, the ureter is reunited by the invagination method of Van Hook. Following this, the operative field is cleaned of blood, and the peritoneum readjusted by a few interrupted sutures about the periphery of the kidney. Special pains should be taken to see that the kidney is replaced high enough toward the liver so that there is no angulation of the vein.

Closure of the Abdomen.—The next step consists in closing the abdomen after the bowels have been replaced. This is made certain by using first a mattress suture of stout silk placed in the linea alba. After tying this, the severed abdominal muscles can easily be approximated in layers. A continuous suture is used for the transversalis, including at the same time the peritoneum. Mattress stitches are used for the recti, while the obliques are held by interrupted sutures. Closure of the subcutaneous tissue and skin completes the operation. Silk is used throughout the operation, both for ties and sutures.

The dog is placed in a metabolism cage so that the urine can be accurately collected and measured.

The operation is a difficult one, but after some experience it was found possible to restore the circulation in about an hour following its interruption. Forty-three dogs were subjected to operation, of which sixteen survived in suitable condition for further physiological observations.

Examination of the Renal Function.

The operated animals were divided into two series. In the first, the function of the reimplanted kidney was compared with that of the intact one, at periods varying from 2 days to 3 weeks after the primary operation. For this purpose the animal was anesthetized with paraldehyde, each ureter brought out onto the flank through

[9] The method of suture used is that elaborated by Carrel. Carrel, A., La technique opératoire des anestomoses vasculaires et la transplantation des viscères, *Lyon méd.*, 1902, xcviii, 859; Anastomosis and Transplantation of Blood Vessels, *Am. Med.*, 1905, x, 284; The Surgery of Blood Vessels, Etc., *Bull. Johns Hopkins Hosp.*, 1907, xviii, 18.

lumbar incisions, and the urines were compared. This method has been described in a previous communication.[10]

The dogs of the second series were subjected to removal of the unoperated kidney at times varying from 5 to 14 days after the primary operation. The work of their remaining, reimplanted kidney was later examined and compared with that of control dogs, in whom a single nephrectomy had been done.

Series I.

Two typical protocols will suffice to show the results of this series.

June 17, 1915. Dog 66. Weight 10,454 gm. Operation at 10 a.m.; reimplantation of right kidney.

June 18. Has made a good recovery. Blood urea is 0.302 mg. per liter.[11] Excretion of phenolsulphonephthalein given intravenously is 51 per cent in 2 hours.[11]

June 19. Given paraldehyde,[11] 1.7 cc. per kilo of body weight, by stomach tube, followed by 200 cc. of water. Ureters exposed in loins, and cannulæ introduced delivering into test-tubes. Carotid blood pressure recorded on kymograph.

11.10 a.m. Urine appears without delay from each side. Collected for 1 hour.

12.10 p.m. 5.0 gm. of sodium chloride in 20 cc. of water given intravenously. Good diuresis from each side; urine collected for 1 hour.

1.10 p.m. Animal killed. The blood pressure during the experiment varied from 128 to 138 mm. of mercury, except just after the injection of the hypertonic salt solution. The sutured vessels were free from obstruction.

Microscopic examination of the reimplanted kidney showed the capsular spaces wide for the most part, and the glomerular tufts containing a considerable amount

---

[10] Quinby, W. C., and Fitz, R., Observations on Renal Function in Acute Experimental Unilateral Nephritis, *Arch. Int. Med.*, 1915, xv, 303.

[11] As was shown in the communication noted above (Quinby and Fitz[10]) the two most dependable tests of renal function are the excretion of phenolsulphonephthalein and the estimation of the blood nitrogen or blood urea. The urease method of Marshall (*Jour. Biol. Chem.*, 1913, xv, 487) was used. By this the normal amount of blood urea of the dog is found to lie between 0.200 and 0.380 or 0.400 mg. per liter. The phenolsulphonephthalein excretion was estimated by the method of Rowntree and Geraghty (*Jour. Pharmacol. and Exper. Therap.*, 1909–10, i, 579). Paraldehyde was used, because in proper dosage it causes neither diuresis nor lowered blood pressure. It must be used fresh, however, because it loses strength on exposure to the air, thus making the dosage uncertain.

of blood. The cells and nuclei of the tubules were well preserved except in a very few areas where a small amount of cellular desquamation had taken place. The blood vessels were everywhere dilated, but no evidence of thrombosis or infarction could be found. A considerable number of hyaline bodies were seen in the collecting tubules, evidently casts. The uninvolved kidney was normal in all respects. Its capsules showed active diuresis, but the blood vessels were not so evidently dilated as were those of the operated organ.

		Amount of urine	Percentage of NaCl	Amount of NaCl*
		cc.		*gm.*
1st hr.				
Before diuresis	Right	5.0	0.90	0.045
	Left	2.5	0.55	0.013
2nd hr.				
After diuresis	Right	60.0	0.95	0.570
	Left	22.0	0.95	0.209

* The chlorides were determined by the Volhard method at first; later by that of McLean and Van Slyke (*Jour. Biol. Chem.*, 1915, xxi, 361).

We see that here the kidney without nerves shows an increased function over the normal one, both before and after diuresis.

June 30, 1915. Dog 67. Weight 11,363 gm. Left kidney reimplanted.

July 12. Has made a good recovery. Urine free from albumin and sugar. Average daily amount, 195 cc. Given paraldehyde, 1.7 cc. per kilo of body weight, followed by 200 cc. of water. Ureters exposed on flanks and cannulated. Blood pressure tracing from carotid.

11.05 a.m. Flow from right kidney begins a little later than from left which started immediately on delivering ureter. Urine collected for 1 hour after flow from each was established.

12.16 p.m. 5.0 gm. of sodium chloride in 20 cc. of water injected intravenously. Marked diuresis.

1.20 p.m. Animal killed. Blood pressure average was 133 mm. of mercury. Renal vessels without trace of clot, though there were a few adhesions about the kidney. A sample of blood drawn just before death showed 0.354 mg. of urea per liter.

Microscopic examination showed that both kidneys were normal in all respects except that in the one previously reimplanted a few mitotic figures could be found in the tubular cells after careful search. These were evidently the sequel of previous cellular degeneration.

		Amount of urine.	Percentage of NaCl.	Amount of NaCl.
		cc.		*gm.*
1st hr.				
Before diuresis	Right	2.5	1.12	0.028
	Left	3.0	0.93	0.028
2nd hr.				
After diuresis	Right	40.0	0.82	0.328
	Left	36.0	0.96	0.345

In this experiment the operated kidney has a function about the same as that of the normal side.

These two protocols fairly represent the findings in this first series which comprised eleven dogs. For a period following operation varying from 10 to 14 days the denervated kidney shows an increased absolute function both for fluid and salt, as compared with the normal kidney. At times this increase is relative as well. This is true of the unstimulated organ, and especially so of the one subjected to the diuretic action of sodium chloride. Beyond this period, however, the balance is regained, so that each organ, operated and intact, divides the labor in very nearly equal parts.

We see here also that absence of the renal nerves abolishes the temporary inhibition of flow so often seen normally after the handling of the ureters necessary for their exposure in the loin. Urine from the denervated side always flowed immediately on section of the ureter; but in some instances the normal side showed an inhibition lasting for as long as 5 minutes. This is analogous to the temporary inhibition occasionally seen on passage of a ureteral catheter in man.

Series II.

The following is a typical protocol.

Nov. 20, 1915. Large male, of Newfoundland type. Weight 15,900 gm. Intratracheal ether and reimplantation of right kidney. Circulation restored after being interrupted for 1 hour.

Nov. 23, 1915. Has made a good recovery; eats well and does not vomit.

Nov. 30, 1915. Has entirely recovered. Wound healed by first intention.

Dec. 4, 1915. Ether. Lateral incision in left flank through which the kidney was removed after ligation of its vessels with silk. Wound closed in layers.

Dec. 7, 1915. Has made an excellent recovery. Passes 320 to 340 cc. of urine daily. Blood urea, 0.456 mg. per liter. Phenolsuphonephthalein, 60 per cent in

2 hours. Urinary sediment shows a rare blood corpuscle; no casts; a few leukocytes. Sugar and albumin absent.

Dec. 11, 1915. Animal well. The 24 hour amount of urine has fallen somewhat and its concentration has increased.

Dec. 14, 1915. Has a rather marked balanitis, and does not urinate until the demand is imperative. After about 48 hours during which no urine was passed, voided a little over 700 cc.

Dec. 18, 1915. Balanitis has responded to irrigation with boric acid and animal is now well. Output of phenolsulphonephthalein, 50 per cent in 1 hour. No albumin or sugar.

Dec. 22, 1915. Intravenous injection of 500 cc. of normal (0.8 per cent) salt solution. Urine withdrawn by catheter at 30 minute intervals showed the following:

	Amount.	NaCl per liter.
	cc.	*gm.*
1st half hr.	83	10.6
2nd " "	62	12.3
3rd " "	48	13.0
4th " "	11	17.2

The urine before diuresis contained 19.10 gm. of sodium chloride per liter. It is thus seen that the kidney responds quickly to diuresis and regains its equilibrium within a normal time limit.

Jan. 31, 1916. Given 2 gm. of lactose intravenously. At the end of 5 hours 1.72 gm. were found in the urine.

Feb. 7, 1916. The 24 hour amount of urine has been measured for 65 days, giving an average of 180 cc. It contains neither albumin nor casts.

Feb. 9, 1916. Blood urea 0.320 mg. per liter. 61 per cent of phenolsulphonephthalein is excreted in 1 hour. Animal seems to be perfectly normal.

Four other animals of this series were killed after having shown normal kidney function for a month or longer. In each instance the kidney showed microscopic evidences of some hypertrophy of the elements, which usually occurs after unilateral nephrectomy. In two instances the kidney also showed a small depressed scar in the cortex with sclerosis of the normal elements and infiltration by connective tissue. These areas seemed to be the result of small focal necroses caused by interruption of the blood supply during operation. They were never of any considerable size, so that the function of the organ remained uninfluenced.

The results of this second series show that the life of dogs having a single reimplanted kidney is maintained in a normal manner, as estimated by renal functional tests as well as by other more general methods of observation.

DISCUSSION.

The experiments of the first series show that the immediate effect of loss of nerve control over the kidney is a period of overaction. This occurs in all cases, and in the presence of apparent health, as judged by the general condition of the animal, by the normal content of the blood in urea, and by a normal output of phenolsulphonephthalein. This period exists for a varying time, but balance has always been restored by the end of 2 weeks. The kidney recently deprived of its nerves is without vasomotor control; the organ is tense and appreciably enlarged; its vessels are dilated, and following the increase of blood flow there is an increase of function over that of the normal organ. Resumption of tone on the part of the blood vessels brings again normal function.

The results here would seem to be analogous to those vasomotor changes occurring in the splanchnic area after section of the cord. Following this operation there occurs a marked dilatation of the mesenteric vessels, but in a short time vasomotor control is again established. Vascular tone may be resumed through the intervention of other more peripheral nerve ganglia, or the smooth muscle fibers of the vessel wall may possibly regain their tone without such intervention. Also, in the kidney there are ganglion cells, especially in the region of the renal sinus, which may be responsible for the resumption of vasomotor control. Although we know that fibers of the sympathetic type are able to regenerate much more quickly than are those of the peripheral nerves, that they should be able to grow to the renal blood vessels and resume control over them within 2 weeks after section seems improbable. Certainly nerve control by the normal pathways could never have been regained in Zaaijer's dog whose kidney was sutured to the iliac vessels, or in those of Lobenhoffer who used the splenic vessels.

The time variation in regaining normal function is probably to be explained by the greater or less degree of surgical insult in the indi-

vidual case. No kidney showed normal function after being excluded from the circulation for longer than 1 hour and 20 minutes.

The second series of observations indicates that a single kidney which has been removed from the body and subsequently reimplanted, can maintain normal life for apparently indefinite periods. Also such a kidney is able to respond to the excessive demands made on it by the injection of various test substances. My results in this group of experiments fairly coincide with those of Lobenhoffer, except in a few details. He found that the 24 hour amount of urine passed by his dogs varied between 1,500 and 2,000 cc. This is quite unusual. Normal cage dogs in a large number of observations made by us, are found to pass from 200 to 450 or 500 cc. of urine daily, having a specific gravity of about 1.030. Of course this is but a rough average, since all our animals had water continuously at hand, and must have taken varying amounts from day to day. Their diet was of meat. I feel, therefore, that the continuous excretion of such large amounts of urine tends to suggest the absence of complete return to normal conditions.

Again, in the three infusion experiments reported by Lobenhoffer, his animals put out amounts of water and salt which varied widely, though the quantities infused were the same. In the few infusions done by me the resulting outputs were all within 10 or 12 per cent of each other.

The observations on the relative values of the different methods used for testing renal function made by Quinby and Fitz[10] showed that the estimation of the blood nitrogen, or that part of it composing the blood urea, and the output of phenolsulphonephthalein, together form the best means of measuring renal function. Further experience with these tests in clinical work by many observers has confirmed this opinion. I have therefore been content to follow the dogs of this second series by means of these tests, rather than by phloridzin or lactose, as did Lobenhoffer.

The above results, though they throw no direct evidence on any possible secretory function of either the vagus or splanchnic nerves, seem to suggest that if this exists it must play a minor and infrequent part. Under all the conditions produced both by my experiments and by those of Lobenhoffer, the denervated kidney has been

seen to react in an entirely normal manner. One may ask, therefore, if secretory nerves to the kidney are assumed to exist, under what conditions they are manifest; for no lack of such action seems to be demonstrable. Added to the inability of the present observations to show any failure of kidney function which might be ascribed to lack of secretory nerve influence, is the recent work of Cow,[12] who finds in the duodenal mucosa some substance which has a definite diuretic effect on the kidney by means of a hormone action.

It is probable that vasomotor conditions in the kidney, added to the chemical and hormone action of substances contained in the circulating blood, will be found entirely adequate to explain all variations and types of normal renal function.

SUMMARY.

1. By means of vascular suture it is possible to remove the dog's kidney from the body and later to restore it to its former position.

2. Such a kidney is removed from the control of the nervous system, at least for a time.

3. Examination of the function of a kidney so treated shows an initial period of overaction, as compared with that of the normal kidney.

4. This is followed by balanced action.

5. The more recent tests of renal function show that a single, reimplanted kidney is able to maintain normal life indefinitely.

6. The results of these experiments, together with the evidence already at hand, suggest strongly that secretory nerves to the kidney do not exist.

EXPLANATION OF PLATES.

Plate 81.

Fig. 1. The transverse abdominal incision extending from the outer border of the left rectus muscle well down into the right flank.

Plate 82.

Fig. 2. The oblique muscles and both recti have been divided and the abdomen is being opened by incision of the transversalis in a direction parallel to its fibers.

---

[12] Cow, D., *Jour. Physiol.*, 1914–15, xlix, 441.

Plate 83.

Fig. 3. The intestines have been withdrawn for the most part from the abdomen, and lie to the left covered by silk handkerchiefs. The kidney, with its vessels and ureter, lies well exposed. Above the kidney is seen a portion of one of the lobes of the liver. At its inner side lie the inferior vena cava, and the pancreas enclosed by a portion of the duodenum.

A METHOD FOR OBTAINING SUSPENSIONS OF LIVING CELLS FROM THE FIXED TISSUES, AND FOR THE PLATING OUT OF INDIVIDUAL CELLS.

By PEYTON ROUS, M.D., and F. S. JONES, V.M.D.
(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 84 to 87.

(Received for publication, January 15, 1916.)

The only cells of the mammalian body which lend themselves as individuals to accurate experimentation *in vitro* while yet alive are the blood cells, the cells of exudates, and the spermatozoa. In saying this we do not overlook the usefulness of tissue cultivation or of experiments with living tissue fragments, of the transplantable tumors for instance. But both means of study involve, not individual cells, but complexes of different cells, which can be standardized only roughly, and which cannot be broken up into their component elements or protected from confusing factors, such, for example, as are introduced by death and autolysis of the central tissue portions. These difficulties have led us to work out a method whereby living tissue cells can be obtained as individuals in suspension, and, if desired, can be plated out in a culture medium (plasma) just as are bacteria. After growth the cells can be liberated again, and again plated successfully.

The method consists, in brief, in the growth of tissue in plasma, according to Carrel and Burrow's modification of Harrison's technique, and the liberation of the new cells by digestion of the clot with trypsin. We had noted that if the serum of a growing tissue culture is replaced with Locke's solution at room temperature the cells of the growing strands that extend out into the medium sometimes contract into spheres, which may be separate or, when growth has been dense, loosely attached, side by side. The general outline of the culture is maintained because the cells are held in place by the fibrin network; and if serum is added and incubation renewed they again put forth processes, and, joining each other, again form strands. The problem

has been to cause the cells to contract and then to liberate them from the fibrin network. This is readily done with trypsin in Locke's solution (Fig. 1); and the resulting suspension can be freed by filtration of all but individual cells.

Method.

We have used the trypsin powders of Merck, Grübler, and Kahlbaum. It is necessary to free them as far as possible from the ammonium sulphate which constitutes the greater part of their bulk. According to Kirchheim,[1] the trypsin of Merck does not contain ammonium sulphate; but we have found it present in as great amount as in the other preparations mentioned. It should be got rid of by Kirchheim's method. The trypsin powder is shaken briefly in absolute alcohol and allowed to stand while the heavy sulphate settles out. The supernatant flocculus is collected on a filter, rapidly washed with ether, dried in the air, and dissolved in Locke's solution (Locke's modification of Ringer's solution, but without sugar). The yield from 2 gm. of the unpurified trypsin is dissolved in 98 cc. of Locke's solution. The cloudy, yellowish fluid is filtered, first through paper, then through a Berkefeld cylinder (N) to sterilize it, and is distributed in test-tubes and kept in the ice box. It loses very slowly its ability to digest and can still be used after 2 months. 3 per cent trypsin digests plasma clots more rapidly and does not harm most cells; but 5 per cent kills cells. Unpurified trypsin powders can be employed but the results are not so good.

The tissue from which cells are to be obtained should be cultivated preferably in plasma diluted with Locke's solution in order that the fibrin network to be digested shall be slight. A mixture of one part of plasma with three of Locke's solution is a medium suitable for most tissues. If there is need for a thick suspension of cells many bits of tissue should be grown. It is convenient to flood them in small Petri dishes with a thin layer of the dilute plasma. After clotting has taken place each dish is sealed to prevent evaporation, and placed in the incubator. A stout cord dipped in hot, sterile paraffin and thrust between the outer and inner rim of the dish, with one end

[1] Kirchheim, L., *Arch. f. exper. Path. u. Pharm.*, 1911, lxvi, 352.

left free, is useful for sealing. A pull on the free end will release the top of the dish.

When growth is established the trypsin solution, warmed to 37°C., is poured on, filling the dish above the plasma, and incubation is continued. In a few minutes some of the tissue fragments are free, and within about an hour the clot has disappeared and there remains a clear fluid containing numerous tissue particles. This is taken up with a pipette, stirred to break up any loose aggregations of cells, diluted with Locke's solution, filtered through sterile gauze, and centrifugalized. The fine, powdery, yellowish gray sediment will consist of discrete cells, nearly all of them alive. They can be washed repeatedly if need be. We prefer for this purpose the "gelatin-Locke's,"—Locke's solution containing $\frac{1}{8}$ per cent of gelatin,—which, as Rous and Turner showed,[2] protects fragile cells against mechanical injury. If the cells are to be plated again in plasma they need not be washed, but after centrifugalization can be suspended in the Locke's solution used to dilute the plasma. Plating is done, as before, in Petri dishes.

Results.

The cells liberated as individuals by trypsin are those which grow out into the medium in strands or a meshwork, or which wander out separately (connective tissue cells, endothelium (?), choroid, sarcoma, and splenic tissue cells). Thus far we have used successfully the tissue of rat and chick embryos, of rat and chicken tumors, and the normal tissue of young rats. Sheets of growing cells (epithelium) are not readily broken up. Whether individual epithelial cells can be liberated in this way is as yet uncertain. But small groups of epithelial cells are obtained, and bits of striated muscle which live for a brief period when plated again.

The individual cells become approximately spherical when in suspension and the nuclei also tend to, though less perfectly. The change in form is especially noteworthy in the case of elements which, when growing in culture, are stellate or of an attenuated spindle shape with an elongated nucleus. When freed, suspended in

[2] Rous, P., and Turner, J. R., *Jour. Exper. Med.*, 1916, xxiii, 219.

serum, and stained, such cells show no trace of the long protoplasmic processes which they had while growing. With Wright's stain certain of them derived from connective tissue and probably of fibroblastic and endothelial origin have a resemblance to the mononuclear series of the blood (Fig. 2). Their cytoplasm is basophilic. Other cells from the same source are three or four times the diameter of any blood element. These morphological features will be taken up in a later paper.

The freed cells, distributed in plasma as separate individuals and incubated, soon put forth processes and assume their original form. Bits of striped muscle from the embryo may round at the ends, thus gaining a leech shape, and put out short processes (Fig. 4). We have not observed them to proliferate. But the spindle-shaped and stellate cells of connective tissue, sarcoma, and the choroid coat of the eye multiply rather rapidly. If the cells are numerous the plate will show at the end of 24 hours a thick mesh- or feltwork consisting of elements once separate which have reached out and joined each other by means of attenuated processes (Fig. 3). The tendency of scattered cells thus to connect with each other again is striking. At the end of 48 hours the number of growing elements is greatly increased, not only by proliferation but by the "waking up" of cells previously spherical. If small masses of cells are present in the culture, as the result of incorrect filtration, growth from them may be almost explosive, each mass resolving itself into elements that radiate in every direction.

The Replating of Cultures.

The limits of the method have not yet been reached. The freed and plated cells can be liberated anew after growth and successfully plated again in fresh plasma. To judge from our results, the process can be repeated indefinitely. Isolated cells of the chick's choroid continue to form pigment after they have been twice liberated with trypsin and twice replated (Fig. 5).

Cells that have been growing in tissue cultures for more than 24 hours when freed and examined in suspension show, as a rule, fat droplets, and corresponding vacuoles when fixed and stained in the

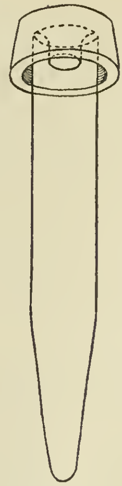
spherical state. Fat droplets have often been noted in tissue cultures and their source is to some extent known.[3] But they are much less prominent in the culture with its extended cells than in the freed, contracted elements. We wish to emphasize the fact that they develop very early, even when growth is taking place in a dilute plasma medium. Only during the first 24, or rarely the first 48 hours, do the cells appear absolutely normal. Later the culture consists for the most part of abnormal elements. This is true also of the freed and plated cells. It follows that replating should be carried out at least every 48 hours.

Technical Difficulties.

The initial cultures must be free from bacteria if the cells are to be replated after their liberation. For the tryptic digestion liberates not only tissue cells but bacterial colonies, and a single one of these latter can by its dispersion ruin all of the new plates. For this reason it is best to cut up the tissue to be grown, in a sterile, glass-sided box, closed with pieces of rubber dam at the ends, through apertures in which the instruments and tissue are introduced, and the hands thrust, encased in sterile, rubber gloves. A small, glass hood with cloth sides will do nearly as well, and it is useful for the replating of cultures. Needless to say a single contamination at any time will ruin a sequence of plates. If the cells are to be used in suspension it is of less importance.

The centrifugalization to bring down tissue cells brings down also fine débris such as bits of cotton, particles of dust, etc., from the fluid. By the time cultures have been twice digested and plated, enough of this will have been collected to mar their appearance, unless special care is taken. Such care consists in the use of well filtered fluids, and centrifuge tubes closed with corks instead of cotton or gauze stoppers. Much time can be saved if the corks are hollowed to fit over the end of the tube, but with a central core to prevent dislodgement (Text-fig. 1). They may be boiled or autoclaved. The central core should be rather short in order that it may remain uncontaminated when the cork is placed on an unsterile surface.

[3] Lambert, R. A., *Jour. Exper. Med.*, 1914, xix, 398.



TEXT-FIG. 1. Centrifuge tube closed with an easily removable cork designed to keep the contents sterile.

SUMMARY.

Individual, living, tissue cells can be obtained in suspension by digesting with trypsin the clot of growing tissue cultures. Under these circumstances the living cells assume a spherical form. When washed and plated in fresh plasma they put out processes and proliferate. After growth in the new plates has occurred the digestion and plating can be repeated. The limits of the method have not yet been reached. We are at work on a number of the problems which it has opened up.

EXPLANATION OF PLATES.

PLATE 84.

FIG. 1. Edge of a culture undergoing digestion with trypsin. The cells have begun to contract into spheres. (Chick embryo.)

PLATE 85.

FIG. 2. Connective tissue and endothelial (?) cells liberated from cultures of the heart muscle and abdominal muscle of a 3 day old rat. Mononuclear cells from the blood of the same animal. Wright's stain. All the cells are drawn to the same magnification.

The cell marked *a* has ingested two red cells. One cell of the muscle series shows vacuoles resulting from a fatty change, and another has attached to it undigested fibrin threads.

PLATE 86.

FIG. 3. Meshwork formed by the anastomosis of connective tissue cells liberated by trypsin and plated as separate individuals. (Chick embryo.)

PLATE 87.

FIG. 4. Striped muscle from a culture incubated 24 hours after liberation by trypsin and replating.

One fragment of muscle, with sharp-cut ends has not grown and has undergone fatty change. But the others give evidence of life, as shown by their change in form, and one has put forth a process. (Rat embryo.)

FIG. 5. Cells from the chick's choroid growing after two liberations with trypsin and two replatings. The formation of pigment is going on actively.

THE PURE CULTIVATION OF SPIROCHÆTA ICTERO-HÆMORRHAGIÆ (INADA).

BY TETSUTA ITO, M.D., AND HARUICHIRO MATSUZAKI, M.D.

(*From the Chiba Medical College and the Chiba Hospital, Chiba, Japan.*)

PLATES 88 AND 89.

(Received for publication, December 1, 1915.)

Numerous theories exist regarding the etiology of Weil's disease. In June 1914 Inada first observed the occurrence of a spirochæta in this disease, which he designated *Spirochæta icterohæmorrhagiæ*. The spirochæta has since been identified as the etiologic agent of Weil's disease. According to Inada[1] the spirochæta does not grow in solid or semisolid culture media except in a slightly modified medium originally employed by Noguchi for the cultivation of the spirochætæ of relapsing fevers, thus necessitating the use of a piece of fresh tissue as one of the ingredients.[2] The pure culture of this organism produces no odor and is invisible to the naked eye on account of the transparency of its colonies. The spirochætæ grown in these cultures are few in number (Table I), although Inada reports that occasionally there were as many as fifty individuals in a field. Judging from the comparatively poor growth of the spirochætæ, it may be assumed that the method employed by Inada is still far from perfect. Contrary to the experience of Inada, we were able to cultivate the organism in solid or semisolid media, and we have succeeded also in obtaining the culture in a fluid medium.

Material for Cultivation.—Guinea pigs are inoculated intraperitoneally with blood or urine from a person suffering from Weil's disease, and after the animals have developed unmistakable symptoms of the disease, *i.e.*, within 3 to 7 days after inoculation, a sufficient amount of blood is withdrawn, under general anesthesia, from the heart of the animals by means of a sterile syringe, the usual aseptic

[1] Inada, R., *Jour. Exper. Med.*, 1916, xxiii, 377.

[2] Noguchi, H., *Jour. Exper. Med.*, 1912, xvi, 199.

precautions being observed. The samples of blood thus obtained are examined under the dark-field microscope for the spirochætæ and those with a positive finding are selected for the purpose of cultivation. The blood may be used at once or after being kept in a sterile test-tube for several days at room temperature, during which time the spirochætæ increase in number in the majority of instances. The cultures are made in a fluid, a semisolid, or a solid medium.

A small piece or an emulsion of the liver or kidney of animals that have died of experimental Weil's disease may also be used for the purpose of cultivation, but we prefer blood from the heart on account of the comparative ease with which other bacteria can be prevented from entering the culture.

TABLE I.

Date.	No. of spirochætæ in one field.	Date.	No. of spirochætæ in one field.
1915		*1915*	
May 14	1– 2	June 15	14–15
" 19	1– 5	" 18	3– 4
" 24	1– 8	" 21	—
" 26	1–18		
June 2	1– 4		
" 4	4–10		
" 8	8–10		
" 12	20–30		

Varieties of Culture Media and Mode of Cultivation.

(*1*) *Semisolid and Solid Media.*—Blood agar and blood gelatin were found to be the most satisfactory media. Blood agar is prepared by mixing one part of the blood from a normal guinea pig or a man with one or two parts of the ordinary nutrient agar while the latter is still in a fluid state at a temperature of about 50°C. The blood gelatin is prepared by mixing one part with two to four parts of the blood at a temperature of about 25-30°C., at which the gelatin is still fluid. As soon as the media are prepared, and before they become solidified, the inoculation is made by adding a drop of the infected blood and distributing it well in the media by stirring the tubes. The inoculated tubes are now placed in a temperature any-

where between 15° and 37°C. As a rule, a layer of fluid paraffin about 2 cm. deep is poured into the culture tubes in order to cover the surface, although an abundant growth may be had without the addition of the paraffin oil. It may be remarked that these blood media appear to be a much deeper red as well as opaque near the bottom, as the erythrocytes gradually sediment before the agar or gelatin becomes solidified.

The culture tubes are best left undisturbed for at least 10 to 14 days. The greater the amount of infected blood introduced into the media, the more certain are the chances of obtaining a culture, for the reason that we thereby introduce more spirochætæ. According to our experience blood gelatin gives a better result than blood agar.

The commencement of growth in the solid media is not constant, but this usually takes about 1 week, after which the spirochætæ increase in number for the following 2 to 3 weeks, at which time the growth reaches its maximum. We therefore renew the culture by transplanting it into the new media every week or two. For this purpose the inoculation is made by the stab method.

The growth of the spirochætæ is accompanied by neither an offensive odor nor by the production of a gas and there is no liquefaction of the media.

Morphology.—The spirochætæ grown in our media show active movement under dark-field illumination (Leitz apparatus) and possess a number of small rectangular curves regularly set along the whole length of the organism. It is difficult to count the curves accurately while the spirochætæ are actively moving, but there are some with from two to three curves and some with as many as fifteen curves. The long specimens resemble *Spirochæta pallida*. The body of the spirochæta presents a granular or beaded appearance due to the unequal refractive power as manifested by different sections, the sections with a stronger refractive power being set alternately with those possessing a weaker power. The average organisms show more than fifteen granules and the size of the granules becomes somewhat smaller towards the extremities, which are drawn out into sharp points. In a resting specimen or before motion has begun one of the ends may assume a blunt appearance.

The length of the small specimens attains a quarter of the diameter of an erythrocyte, while that of the long ones is five times as long.

Motility.—The movements are sometimes forward, sometimes backward, and at times certain lateral motions are also noticed. The organism may shoot through the field with great rapidity or display a corkscrew or serpentine movement along the long axis. In a fresh preparation sealed with dammar and kept at room temperature, the spirochætæ become gradually sluggish and finally immobile within varying lengths of time, occasional mobile individuals still being discernible among them.

Number of Spirochætæ.—The number of organisms in a culture may vary according to the age of the latter, but they are generally innumerable, as they gather diffusely or in bundles, and it is impossible to count all the spirochætæ in a field (Fig. 1).

Staining Reaction.—The organisms do not take any of the ordinary aniline dyes, but assume a pinkish or pinkish purple color when stained with Giemsa's solution. The appearance of the stained spirochætæ differs considerably from the morphological features of living specimens (Fig. 2). They are much heavier near the middle and taper off into sharp points at both ends, thus resembling *Spirochæta refringens.* The spirochætæ in a stained preparation are much shorter than the organisms observed under the dark-field microscope. The organisms grow at any temperature between 15° and 37°C., but the optimum temperature is between 20° and 25°C.

Mode of Development.—In a solid culture where the inoculation of the spirochætæ is made in a fluid state the growth is diffuse throughout the media, but in a stab culture they multiply around the stab canal and then spread diffusely. In a young culture the spirochætæ are short and are found near erythrocytes or sometimes attached to them. As time passes the organisms grow longer and wander away from the red corpuscles to form masses or remain scattered. We are unable to decide whether the spirochætæ multiply by longitudinal or transverse division, but we have seen two specimens intertwined. In some instances two spirochætæ are seen to be united at one end, while a bundle of immobile specimens lying parallel may be seen to break up suddenly into units and to move away individually.

(2) *Fluid Media.*—Blood serum of man or ox diluted with an equal part of distilled water or undiluted ascitic fluid or pleural exudate is

sterilized by subjecting it to a temperature of 50–60°C. for half an hour for several successive days. Sterile tubes are each filled with 10 cc. of the fluid. Another way of preparing a fluid medium is to follow the method of Noguchi; namely, to add to the above fluid a small piece of kidney from a normal guinea pig and then to use the media after ascertaining the sterility by incubating them for 24 hours. Instead of the kidney, small amounts of coagulum of human or guinea pig blood may be used.

The inoculation of the fluid media is made by introducing one or two loopfuls of the infected blood containing the spirochætæ. The tubes are then placed in a temperature varying from 15–37°C. By this method we have succeeded in obtaining a good growth which was first noticed after 3 to 10 days by the appearance in the clear fluid of a light haze resembling a culture of *Spirochæta pallida*. Upon examination under the microscope numerous spirochætæ identical with those grown in a solid medium were found. Unfortunately, the culture died out in the second generation, probably owing to contamination with a bacillus of the *coli* type or to the lack of the red corpuscles in the fluid media. The transfer in this case was made on the 5th day and the spirochætæ died on the 5th day of the second generation.

A pleural exudate rich in fibrin seems to be the most suitable fluid medium for the purpose of cultivating this organism.

Pathogenicity.—For the purpose of determining the pathogenic property of the pure culture of the spirochæta we have inoculated a small quantity of the culture into the peritoneal cavity of a number of guinea pigs. In the course of 4 to 8 days after the inoculation the animals succumbed after the usual symptoms of the disease. The post-mortem showed all the characteristic lesions. From these animals we have recovered the same organism in pure culture. This experiment completes the links of evidence proving that the spirochæta in question is the causative agent of Weil's disease, and it shows that the pathogenicity of the organism is not noticeably diminished through artificial cultivation. The spirochætæ were found in sections of the liver of the guinea pig from which the culture was derived and of the guinea pig which showed the typical symptoms after receiving the inoculation of the culture (Figs. 3 and 4).

According to Ashizawa's experiment, blood serum from a patient once attacked by Weil's disease has a slight bactericidal action upon the spirochætæ cultivated by our methods.

CONCLUSIONS.

Pure cultures of the spirochætal causative agent of the disease known as Weil's disease, or febrile icterus, in Japan, have been obtained by us in a solid, a semisolid, and a fluid medium. The spirochæta thus isolated remains pathogenic for guinea pigs for many generations. Up to the present time we have succeeded through the courtesy of Professor Nagayo, Dr. Konuma, and Dr. Ishihara, in cultivating three different strains.

The spirochæta is a facultative anaerobe.

The solid and semisolid culture media possess one disadvantage, in that they are opaque on account of the addition of red blood corpuscles; but it is hoped that this drawback may soon be overcome by further studies. We shall report later the results of investigations regarding various questions in immunity as well as further details regarding the biological properties of the spirochæta.

We wish to express our gratitude for the many valuable suggestions and the assistance which Professor Dohi and Dr. Noguchi rendered us during the execution of the present work.

EXPLANATION OF PLATES.

Plate 88.

Fig. 1. Dark-field view of *Spirochæta icterohæmorrhagiæ* from a pure culture in a semisolid blood gelatin medium. The dark spheroid bodies with a refractive ring are erythrocytes. The white, wavy, beaded lines represent the spirochætæ. Semischematic.

Fig. 2. A film preparation of *Spirochæta icterohæmorrhagiæ* from a pure culture in a semisolid blood gelatin medium. Giemsa's stain. Semischematic.

Plate 89.

Fig. 3. Distribution of *Spirochæta icterohæmorrhagiæ* in the liver of a guinea pig in which typical symptoms and lesions had been produced by injecting a pure culture of the organism. Levaditi silver impregnation method. × 1,000.

Fig. 4. A film preparation of liver emulsion obtained from a guinea pig which died of experimental Weil's disease produced by a pure culture on the sixth day. Giemsa's stain. × 1,000.

THE BACTERICIDAL PROPERTIES OF THE QUATERNARY SALTS OF HEXAMETHYLENETETRAMINE.

I. The Problem of the Chemotherapy of Experimental Bacterial Infections.

By WALTER A. JACOBS, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 1, 1916.)

The researches of Ehrlich and his coworkers during the past decade, which led to the discovery of salvarsan, have created a new procedure in the search for remedies for the control of infectious diseases. The discovery of this drug was the result of a logically conducted development of the chemistry of organic arsenic compounds in the directions indicated by their biological properties. This new procedure may be regarded in a way as a revival of the one in use during the time preceding the period of purely biological method, for at that time the therapeutic values of quinine, mercury, arsenic, and salicylic acid were first discovered. The resemblance of the old to the new consists essentially in the fact that in each case chemical substances were used as the therapeutic agents, with the difference, however, that the finding of the old chemical remedies in no way involved the use of synthetic organic chemistry, controlled by constant biological cooperation, the essential feature of the new chemotherapeutic method. This new departure must not be confused with the older synthetic pharmacology, the aim of which was quite different.

A few workers, and the number is constantly increasing, have been attracted to the new field, and the occasional report of promising results, taken with the drugs already proven of therapeutic value, affords ample justification for greater expectations. But to each worker the first and perhaps greatest problem which presents itself is the finding of leads. Salvarsan, optochin, and the trypan dyes, the chief products of the new chemotherapy, all owe their discovery to the development of leads which had been furnished by clinical observations in the

field. Arsenic, mercury, quinine, and certain dyes are perhaps the only substances which up to now have been regarded with assurance as leads for chemotherapeutic development. And of these, with the exception of optochin and perhaps of salicylic acid, all have been indicated for protozoan infections only, and there is promise that by the proper development of these substances the problem of the control of most protozoan infections will be successfully solved.

When we turn, however, to the cure of infections of purely bacterial origin the problem which confronts us is found to be more difficult of attack. Here not only the difficulty of procuring a suitable experimental infection as a test object, but the absence of clinical observations which might be regarded in the nature of leads which could form the basis for a rational chemotherapeutic procedure, makes it imperative from the start to seek for such leads. The successful chemical development of quinine which led to the discovery of ethylhydrocuprein already indicates that resource may be had eventually to the above mentioned protozoan leads. And it is a fact that perhaps with the exception of salicylic acid this drug is the first substance to have been used successfully in an experimental bacterial infection. This one fact creates the hope that chemotherapy may find a wider application in the control of bacterial infections. It is conceivable, however, that ultimate success may depend upon the finding of leads other than those which have been successfully used against protozoan infections.

It would seem that a wealth of material should lie ready among the numerous classes of organic substances which have been found to exert powerful bactericidal effects *in vitro*. But the failure of many of these when tried in experimental infections has led to the realization that besides a bacteria-killing property the fulfillment of certain other requirements is essential for the achievement of an internal antisepsis. Some workers believe that no indication of the probable effect of a substance *in vivo* can be discerned from its action *in vitro*, and that the successful control of an infection by chemical agents can be attained only by indirect means. This might occur, on the one hand, as perhaps in the case of atoxyl, by the chemical transformation *in vivo* of the injected drug into some active form. On the other hand, the substance might act through the protective mechanism of

the host either by increasing phagocytosis or by stimulating the production of immunity principles. It is, of course, probable that in some instances these phenomena might play a part and that a possible scheme of chemotherapeutic attack could be developed along these lines.

Owing to the ease of handling bacteria *in vitro* and the simplicity of the bactericidal tests it would be unwise because of former failures to condemn the *in vitro* method, at least as a means of initial orientation. If it were accompanied by certain parallel studies the *in vitro* method should do more than afford only orientating data. As pointed out by Bechhold and Ehrlich[1] and others in the past, besides a mere bactericidal power other conditions must be fulfilled before a substance may be considered even a possibility as a therapeutic agent, provided of course a direct action by the drug itself is in question.

Aside from the obvious conditions of solubility and relative non-toxicity, the drug once in the circulation, whether by direct intravenous injection or by absorption, must be maintained therein for a sufficient length of time and in sufficient concentration in order to unfold its *in vitro* effect. In other words, its free access to the foci of infection should not be completely obstructed.

To accomplish this it must not enter too rapidly into chemical or physical combination with the constituents of the tissues, of which the blood is a fair representative. It must not be too speedily eliminated. And, finally, it must not be too rapidly altered in any way by metabolic processes which would nullify its bactericidal character. There may be still other and less definite factors which separate the *in vitro* from the desired *in vivo* result. If, however, the *in vitro* bactericidal tests could be complemented by a parallel study of those properties of substances which would decide whether they could satisfy the above requirements *in vivo*, our choice of substances for the *in vivo* experiments could be in great measure controlled. A system, though somewhat arbitrary, would be substituted in an undertaking which would otherwise be directed by a haphazard and entirely opportunistic policy.

These considerations have convinced us that the procedure in the search for leads in the chemotherapy of bacterial infections may be

[1] Bechhold, H., and Ehrlich, P., *Ztschr. f. physiol. Chem.*, 1906, xlvii, 173.

logically systematized as follows: Substances which either by their general structure or by the possession of characteristic atomic groups are representative of as many types of organic substances as possible should be systematically selected for bacteriological and biological testing. Such facts as the bactericidal power and partial specificity for certain types, compatibility with tissue constituents (serum), and resistance to profound and rapid metabolic alteration should be noted and considered in the final interpretation of what in the chemical constitution of the substances is responsible for the observed biological behavior.

With organic substances there will be considerable difficulty in satisfying the last requirement. In the case of arsenicals and mercurials it is immaterial whether metabolization should occur, for the therapeutic characteristics of such compounds are elements. Their value may partly depend upon such metabolization. It does not seem improbable, however, that bactericidal substances may be found which, even though to a less degree than the arsenicals and mercurials, may be sufficiently resistant to metabolic changes to enable them to produce a sterilizing effect before they are disposed of by the host. The large number of pharmacologically active preparations must all persist long enough after administration to produce their physiological actions.

From the representative substances which have been found to possess the required biological properties, two classes of leads might be obtained: first, those substances which, like quinine, owe their bactericidal action to the general structure of the molecule; and, second, those which, like phenol, are bactericidal principally because of the possession of a certain atomic group. Once in the possession of *bactericidogenic*,[2] tissue-compatible molecules or side-chains, the same systematic development so successfully employed in the development of organic arsenicals by the alteration or addition of groups to the molecule might be here repeated in order to augment the specific bactericidal action, to detoxify it, or in some other way

[2] The word *bactericidogenic*, of obvious derivation, is employed in this and the following articles as a convenient term to express the property of certain chemical groups, when introduced into an organic molecule, of imparting bactericidal properties to that molecule.

furnish it with biologically desirable properties. In this way substances could be obtained which would form a rational basis for chemotherapeutic investigations.

The problem of the chemotherapy of bacterial infections and a possible scheme for its systematic attack have been discussed above in some detail with the purpose of affording a basis for a better understanding of the material which will be presented in the following papers.

From its nature this material will touch on but one phase of the above scheme and no claim is made of its complete realization. We shall present the results obtained in a systematic attempt to alter chemically the molecule of hexamethylenetetramine with the object of obtaining a class of bactericidal substances which could be employed in experimental infections. The use of this drug was inspired by the interest felt by Dr. Flexner in the possible application of some of its derivatives in the treatment of experimental poliomyelitis, and the material which will here be presented is but a part of a larger undertaking executed with it.

We shall attempt to show how, by the selection of a certain molecular group, namely hexamethylenetetramine, it has been possible to demonstrate its general bactericidogenic character. By the combination of this substance in the form of quaternary salts, in the manner to be described later, with a great variety of other molecular groupings a new class of bactericidal substances has been prepared[3] in which the bactericidal nature was principally attributable to the hexamethylenetetramine nucleus. On the other hand, the degree of this action was determined by the nature of the molecular groups added to hexamethylenetetramine. These added groups were likewise responsible for the partial specificity of certain of the preparations for particular bacterial species. This partial specificity did not favor one species alone, but all the species tested were found to be separately and specifically susceptible to some particular type of hexamethylenetetramine derivative. We must therefore conclude that the bactericidogenic character of hexamethylenetetramine ex-

[3] For the chemistry of these substances and the references to those prepared by others see Jacobs, W. A., and Heidelberger, M., *Jour. Biol. Chem.*, 1915, xx, 659, 685; 1915, xxi, 103, 145, 403, 439, 455, 465.

hibited in its quaternary salts is not specific but general in character. The specificity, however, is furnished by the proper choice of the molecular grouping added.

It will also be shown that a few of the hexamethylenetetraminium compounds which were tested were either not at all or but slightly inhibited by serum. A few, on the other hand, were found to be greatly inhibited by serum. The fact, however, that any one of the hexamethylenetetraminium salts is compatible with serum is enough to demonstrate the serum compatibility of the bactericidogenic hexamethylenetetramine portion of the molecule itself. We have here, therefore, a bactericidogenic, serum-compatible group. The remainder of the molecule determines the serum incompatibility of those substances the action of which was found to be inhibited by serum.

In the same way the toxicity relationships were found to be determined by the groups contained in that portion of the molecule added to the hexamethylenetetramine nucleus.

We can regard the material here presented merely as a beginning, but we feel that such a treatment of the problem as here presented may ultimately result in an accumulation of data which will be of value in the systematic search for substances which may be used in the control of experimental bacterial infections. Before passing judgment, however, on the chances offered by the further development of the quaternary salts of hexamethylenetetramine, the behavior of these substances in the animal organism should be studied in order to determine whether the bactericidogenic group in itself is sufficiently resistant towards metabolic changes. Otherwise these compounds as a class would be bactericidally inert *in vivo*.

THE BACTERICIDAL PROPERTIES OF THE QUATERNARY SALTS OF HEXAMETHYLENETETRAMINE.

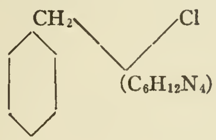
II. The Relation between Constitution and Bactericidal Action in the Substituted Benzylhexamethylenetetraminium Salts.

By WALTER A. JACOBS, Ph.D., MICHAEL HEIDELBERGER, Ph.D., and HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 1, 1916.)

Hexamethylenetetramine, as a tertiary nitrogen compound, reacts readily with benzyl chloride, bromide, or iodide, and their numerous nucleus substituted derivatives to form quaternary salts. The results obtained in the study of the bactericidal properties of such substances are the subject of the present communication. In these compounds, the structure of which may be represented as follows,



it is seen that by means of a CH_2 side-chain the hexamethylenetetramine molecule is linked to a benzene nucleus. By the use of a great variety of substituted benzyl halides it was found possible to prepare for study a variety of hexamethylenetetraminium salts[1] in which the benzene nucleus was varied at will in the character, number, and position of the different atoms and groups introduced. By this procedure the opportunity was afforded of studying the effect of chemical constitution upon bactericidal action in a uniform series of substances.

Because the number of substances involved in the investigations

[1] For the chemistry of these substances and the references to those prepared by others see Jacobs, W. A., and Heidelberger, M., *Jour. Biol. Chem.*, 1915, xx, 659; 1915, xxi, 465.

described in these papers was so large, it was found necessary for the practical execution of the bactericidal tests to adopt a scheme which would involve the least amount of work and still furnish a satisfactory measure of the activity of the substances. For this reason the drug dilutions, which as a rule started with 1: 200, were doubled in each successive dilution so that the series 1: 200, 1: 400, 1: 800, 1: 1,600, etc., were the concentrations with which the observations were made. With this scheme it is seen that as the dilutions increase the differences between them become greater, making it possible to regard the figures obtained only as rough approximations to the true values. In the strictest sense account should be taken of the molecular weights of the substances in a direct comparison of their bactericidal properties, but with the dilution scheme here employed this was deemed unnecessary.

In spite of the crudity of our figures it will be seen that certain relationships between the constitution and the bactericidal action are plainly in evidence. The results given below clearly demonstrate that by the addition of hexamethylenetetramine to benzyl chloride a bactericidal substance is obtained, and that by the substitution in the benzene nucleus of different atoms and groups this action may be altered at will, the resulting effect depending upon the number, character, and position of these substituents. In this class of compounds we possess a new group of bactericidal substances in which the hexamethylenetetramine nucleus is directly responsible for their bactericidal character.

EXPERIMENTAL PART.[2]

Technique.—A strain of *Bacillus typhosus* which had been growing on artificial media for several years and which is a good agglutinator was used in testing the germicidal effects of the compounds.

0.5 or 1 per cent solutions of the substance to be tested were made up in physiological salt solution and filtered immediately through a Berkefeld N filter. With sterile salt solution the dilutions of 1: 200, 1: 400, 1: 800, 1: 1,600, 1: 3,200, 1: 6,400, and 1: 12,800 were made and

[2] Some of the tests with *B. typhosus* were performed by Dr. Paul F. Clark, now of the University of Wisconsin, to whom we are greatly indebted for furnishing us with the results of his experiments.

all brought to a temperature of 37°C. To 4 cc. of each dilution, there was added 0.5 cc. of a 24 hour broth culture of *Bacillus typhosus*, and the tubes were put into an incubator or water bath at 37°C. for 4 hours. At the end of this time, one small loopful was taken from each tube and plated in plain agar. It was found necessary to incubate the plates for 48 hours before counting, because some of the colonies did not grow out in 24 hours. Control plates under the same conditions usually contained about 1,000 colonies. Duplicates were run in each case.

Table I presents the bactericidal results obtained with the different preparations tested upon *Bacillus typhosus* by the technique described above. As stated in the introduction, the number of preparations tested and the pressure of other work rendered impossible a detailed study of each substance employed in the tests, so that the figures here given can be regarded only as approximations to the true bactericidal powers of the substances in question. In most instances the figures as given are, if anything, too low. A consideration of the scheme of dilutions employed will show how great the underestimation of the true bactericidal power might be. For example, in the case of the *o*-nitrobenzylhexamethylenetetraminium chloride, given in Table I, the greatest dilution in which this substance killed all the bacteria in 4 hours was 1: 3,200. The next higher dilution tried was 1: 6,400 and this was found ineffective. But if this compound could really kill in a dilution of 1: 5,000 or even 1: 6,000, the value obtained as a result of the scheme of dilution used would be only 1: 3,200. For this reason we must regard the values given only as relative. In spite of this, the alteration in character or position of the substituents in the nucleus was accompanied by changes in the bactericidal action which were too marked to be masked by the dilution scheme employed.

The tests with hexamethylenetetramine itself and the simple aliphatic quaternary salt methylhexamethylenetetraminium iodide showed them to be devoid of action in a dilution of 1: 200. By the substitution in the latter compound of the methyl by the benzyl group the customary influence of the aromatic nucleus was observed. Although not a strong bactericide, the benzyl salt was found to kill all the bacteria present in a dilution of 1: 200. This bactericidal

TABLE I.

Substance.	Killed *B. typhosus* in 4 hrs. at 37°C. in a dilution of 1:
Hexamethylenetetramine	+*
Methylhexamethylenetetraminium iodide	+
Benzylhexamethylenetetraminium chloride	200
o-methylbenzylhexamethylenetetraminium chloride	3,200
m- " "	800
p- " "	800
3, 5-dimethylbenzylhexamethylenetetraminium chloride	400
o-chlorobenzylhexamethylenetetraminium "	1,600
p- " "	800
o-bromobenzylhexamethylenetetraminium "	1,600
p- " "	200
o-iodobenzylhexamethylenetetraminium bromide	1,600
p- " "	1,600
o-cyanobenzylhexamethylenetetraminium chloride	3,200
p- " "	400
o-nitrobenzylhexamethylenetetraminium "	3,200
m- " "	400
p- " "	1,600
2, 4-dinitrobenzylhexamethylenetetraminium "	3,200
o-methoxybenzylhexamethylenetetraminium "	+
p- " "	200
2, 3-dimethoxybenzylhexamethylenetetraminium chloride	+
3, 4- " "	200
3, 4-methylenedioxybenzylhexamethylenetetraminium chloride	200
5-nitro-2-methoxybenzylhexamethylenetetraminium "	400
3-nitro-4- " "	800
2-nitro-3, 4-dimethoxybenzylhexamethylenetetraminium "	3,200
2-acetoxy-3, 5-dibromobenzylhexamethylenetetraminium bromide	1,600
4-acetoxy-3, 5- " "	1,600
2-acetoxy-3, 5-dimethyl-4, 6-dibromobenzylhexamethylenetetraminium bromide	1,600
2-acetoxy-3, 5-dimethylbenzylhexamethylenetetraminium chloride	+
3-carboxy-4-oxybenzylhexamethylenetetraminium "	1,600
3-carbomethoxy-4-oxybenzylhexamethylenetetraminium "	400
2-methoxy-5-carboxybenzylhexamethylenetetraminium "	400
2-methoxy-5-carbomethoxybenzylhexamethylenetetraminium "	400
o-acetaminobenzylhexamethylenetetraminium chloride	800
p- " "	+
1, 2-xylylenedihexamethylenetetraminium dichloride	12,800
1, 3- " "	6,400
Mesitylylenedihexamethylenetetraminium "	12,800

* + indicates growth after exposure to a dilution of 1: 200.

power was further developed by the introduction into the nucleus of various atoms and groups, resulting in the series of substances given in Table I. A study of these brings out the following relationships.

The methyl, chlorine, bromine, iodine, cyano, and nitro groups were all found to increase the bactericidal power of the parent unsubstituted benzyl compound. This behavior of the alkyl, halogen, and nitro group has been frequently observed with other types of organic bactericides; for instance, in the case of the phenols. However, this effect may by no means be regarded as inevitable, as there are many bactericidal substances the power of which is in no way influenced by the introduction of these groups. Examples of this will be found among other types of hexamethylenetetraminium salts to be described in the following paper.

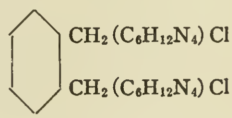
A perusal of the table will show the interesting influence of the position of the substituent upon the bactericidal action. In most cases the *ortho* substituents were found to be more active than either the *meta* or *para* compounds. The regularity of this rule, at least as far as the present series was extended, is striking. The *ortho* methyl, chloro, bromo, nitro, and cyanobenzyl salts were all more active than their other position-isomers.

With this type of hexamethylenetetraminium salt the methoxy group is, on the whole, without influence on the bactericidal effect, if indeed not detrimental. In those compounds which contain the nitro group besides the methoxy group the influence of the former appears to dominate. The 2-nitro-3,4-dimethoxybenzyl derivative was found to equal in effectiveness the 2-nitrobenzylhexamethylenetetraminium chloride. In the same way the 3-nitro-4-methoxy compound was bactericidally about as powerful as the 3-nitrobenzyl salt.

The optimum effect in varying substituents was apparently obtained with the mono- substituted benzyl compounds. In the few cases in which the dimethyl, dibromo, and dinitro derivatives were made, it was found that no advantage as regards the bactericidal value was to be gained, as a rule, by the multiplication of groups. At the same time the greater the number of substituents, particularly in the case of the nitro and halogen compounds, the less was the solubility in water.

In one direction, however, at least when *Bacillus typhosus* was used

as the test object, it was found that a distinct advantage was to be gained by the multiplication of groups; namely, in those compounds in which hexamethylenetetramine was introduced twice into the side-chains. Such salts were obtained by the addition of two molecules of hexamethylenetetramine to ω_1,ω_2-dichloro-*o*-xylene, ω_1,ω_3-dichloro-*m*-xylene, and ω_1,ω_3-dichloromesitylene. These substances possess the following structural formula, in which, of course, the relative positions of the side-chains are different in each case.



These compounds are the strongest bactericides of this group.

Here it should be emphasized that the bactericidal results obtained with this group of substances refer only to their behavior towards *Bacillus typhosus*. As the work developed, other species of bacteria were made the object of an occasional test, but owing to the incompleteness of the results obtained, and to the fact that the technique was varied, it has not been deemed advisable to enlarge on these results in the present paper. It may, however, be said that in general this class of substances was considerably less effective against the streptococcus and meningococcus, but that the results with the gonococcus approached those obtained with *Bacillus typhosus*. This particular group of hexamethylenetetraminium salts cannot, therefore, be regarded as general disinfectants. As a matter of fact, there are but few, if any, organic bactericides which act uniformly against all species or strains of bacteria. A few cases have been selected in Table II to afford a comparison of the effects of several of these salts upon different microorganisms. With the streptococcus, meningococcus, and gonococcus the technique was altered, the time of exposure of the bacteria to the drug being changed to 3 hours and the temperature to 20°. Such a change in technique should, of course, alter the results, but our experience has shown that this rarely exceeded the space of one whole dilution. It is seen from the table that the nature of the substance used determined the effect upon a particular microorganism. A constant relation between the resistances of the various types of organisms is out of the question. The far greater effectiveness

of the two dihexamethylenetetraminium salts against *Bacillus typhosus* is striking. These substances may be classed as "partially specific" for this species.

TABLE II.

Substance.	Killed *B. typhosus* at 37°C. in 4 hrs. in dilution of 1:	Killed streptococcus at 20°C. in 3 hrs. in dilution of 1:	Killed meningococcus at 20°C. in 3 hrs. in dilution of 1:	Killed gonococcus at 20°C. in 3 hrs. in dilution of 1:
Benzylhexamethylenetetraminium chloride	200	+	400	800
p-methylbenzylhexamethylenetetraminium chloride	800	+		
o-bromobenzylhexamethylenetetraminium chloride	1,600	+	+	1,600–3,200
o-cyanobenzylhexamethylenetetraminium chloride	3,200		400	1,600
p-cyanobenzylhexamethylenetetraminium chloride	400		400	800
o-methoxybenzylhexamethylenetetraminium chloride	+*	+	200	800
3, 4-methylenedioxybenzylhexamethylenetetraminium chloride	200	+	1,600	800
o-nitrobenzylhexamethylenetetraminium chloride	3,200	3,200	800	800
m-nitrobenzylhexamethylenetetraminium chloride	400		1,600	1,600
2-acetoxy-3, 5-dibromobenzylhexamethylenetetraminium bromide	1,600	3,200	800	800
2-acetoxy-3, 5-dimethylbenzylhexamethylenetetraminium chloride	+	1,600	800	800
m-xylylenedihexamethylenetetraminium dichloride	6,400	200	+	1,600
Mesitylylenedihexamethylenetetraminium dichloride	12,800	+	400	400

* + indicates growth after exposure to a dilution of 1:200.

From the consideration of the above observations we feel justified in attributing essentially to the hexamethylenetetraminium group the property of determining the bactericidal character of this class of compounds. For direct comparison with another basic side-chain *p*-nitrobenzylpyridinium chloride was prepared. This was found to be ineffective towards *Bacillus typhosus* even in a concentration of 1:200

after 4 hours' contact. The corresponding hexamethylenetetramine quaternary salt killed *Bacillus typhosus* in a dilution of 1:1,600 in 4 hours.

The function, however, of determining the extent and character of this bactericidal property must be attributed to the substituting groups and to the positions occupied by them in the benzene nucleus to which the hexamethylenetetramine is linked. Our experience has shown that such groups likewise decide other biological properties of this class of substances. Without stopping here to deal at length with the toxicity experiments it may be said that in general the toxicity of these compounds is determined by such groups. For example, whereas the *o*-nitro benzyl derivative could be given to mice intravenously in amounts up to 500 mg. per kilo, the 2,3-dimethoxybenzyl derivative was found to be fatal in 0.1 of this dose.

SUMMARY.

By the addition of substituted benzyl halides to hexamethylenetetramine, a series of quaternary salts of this base was obtained. These salts represent a new group of organic bactericides. The results obtained in the tests with these substances upon *Bacillus typhosus* have demonstrated the existence of direct relationships between chemical constitution and bactericidal action within the series.

The bactericidal character is directly attributable to the presence of the hexamethylenetetramine nucleus. The degree of the bactericidal action, however, is determined by the position, character, and number of the groups substituted in the benzene nucleus.

By the introduction of the methyl, chlorine, bromine, iodine, cyano, and nitro groups into the benzene nucleus of the parent benzyl hexamethylenetetraminium salt, the bactericidal power of this compound was notably enhanced. The substitution of these groups in the *ortho* position almost invariably resulted in substances which were more active than their *meta* or *para* isomers. The introduction of the methoxy group was without marked effect.

Several substances in which two hexamethylenetetraminium side-chains occurred were found to be the most active of the substances of this series when tested against *Bacillus typhosus*. Comparative tests with other bacterial types demonstrated that these compounds possessed a marked degree of specificity for *Bacillus typhosus*.

THE BACTERICIDAL PROPERTIES OF THE QUATERNARY SALTS OF HEXAMETHYLENETETRAMINE.

III. The Relation between Constitution and Bactericidal Action in the Quaternary Salts Obtained from Halogenacetyl Compounds.

By WALTER A. JACOBS, Ph.D., MICHAEL HEIDELBERGER, Ph.D., and CARROLL G. BULL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 1, 1916.)

In the course of the biological investigations of the substituted benzyl quaternary salts of hexamethylenetetramine discussed in the previous paper, it was found that the further extension of this chemical type was no longer advisable, in spite of the fact that within this group substances had been found which possessed considerable bactericidal power against *Bacillus typhosus*. This was due partly to the inaccessibility or insolubility of the further preparations of this type which seemed theoretically indicated. Our attention was in consequence turned to the problem of finding a new scheme of chemical procedure which would furnish the possibility of new leads and of broader development. This was found in the readiness with which hexamethylenetetramine as a tertiary base reacted with halogenacetyl derivatives to form soluble quaternary salts with the following general structure,

$$\left.\begin{matrix} R \cdot OC \cdot CH_2 \\ Cl \end{matrix}\right\rangle (C_6H_{12}N_4)$$

in which R might represent the radical of any aliphatic or aromatic primary or secondary amine R′NH, R'_2N, R′R″N, or of any alcohol or hydrocarbon. Because of the general nature of this reaction and the practically limitless number of chemical possibilities offered, it was found possible to furnish the most varied chemical groupings

with the hexamethylenetetramine molecule by the use of the halogen-acetyl side-chain.[1]

Without anticipating the detailed discussion of the observations to be found in the experimental part, the following remarks may be taken to sum up the principal results of the work presented in this communication.

As a result of experiments on a large number of drugs of this type, the general statement may be made that hexamethylenetetramine, when combined with halogenacetyl compounds in the form of quaternary salts, gives rise to a new group of organic bactericides. Hexamethylenetetramine may, therefore, be described as a definite bactericidogenic group. The extent of the bactericidal power of these substances is, in a measure, controlled by the general character of the molecule or of the particular groups contained therein. In addition, the employment of several species of microorganisms has served the purpose of bringing out many examples of partial specificity, at least one substance with a high degree of specificity being found for each of the four species of bacteria used.

The fact that this specificity shifted from one group of bacteria to another with the change in the chemical composition of the radical added to the hexamethylenetetramine, demonstrated that, in contradistinction to the bactericidogenic character, the specificity relations were determined, not by the hexamethylenetetramine nucleus, but by the added radicals.

From the facts set forth above it will be seen that the program presented in the introductory paper has been partially fulfilled. Thus it was found possible, by starting with the molecular grouping furnished by hexamethylenetetramine, to add the most varied atomic groupings with the aid of the $-CH_2CO-$ group, which served merely as a connecting link. The chemical differences in these added groups caused the wide variations observed in the bacteriological results.

In these observations, which must be regarded only as a beginning, we thus see that once in the possession of a biologically active or potentially active molecular group, it is possible, without produc-

[1] For the chemistry of these substances and the reference to those prepared by others see Jacobs, W. A., and Heidelberger, M., *Jour. Biol. Chem.*, 1915, xx, 685; 1915, xxi, 103, 145, 403, 439, 455, 465.

ing profound chemical changes in the molecule itself, to equip it with a reactive side-chain which in turn will react with other molecular groups and which will furnish the necessary biological properties.

In the present paper we wish to present the results of the bactericidal tests performed with these preparations upon *Bacillus typhosus*, streptococcus, meningococcus, and gonococcus. Here, as in the previous communication, the large number of tests which were made necessitated the early adoption of a rough scheme of drug dilutions. For this reason what was said in the former paper regarding the accuracy of the results must be reiterated here. At best the figures given may be considered as rough approximations. In spite of this, however, in many instances pronounced evidence of the influence of constitution on bactericidal action appeared.

EXPERIMENTAL PART.[2]

Technique.—In testing the germicidal efficiency of the compounds to be described below, 0.5 or 1 per cent solutions of each substance, according to the solubility, were made in distilled water and filtered through a Berkefeld N filter. The other dilutions were prepared from this stock solution by the use of sterile distilled water as a diluent.

The series 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400, and 1:12,800 was employed for the tests. In some cases the sparing solubility of the substance necessitated the omission of the lower dilutions.

5 cc. of each dilution were placed in wide mouthed tubes and the temperature was brought to 20°C. To each of these tubes the bacterial suspensions were added.

In the case of the *Bacillus typhosus* 0.1 cc. of a 24 hour broth culture was added to each of the tubes containing the dilutions of the compound. After 3 hours a standard 4 mm. loop of the mixture was plated in order to determine the number of living organisms. The plates were incubated for 48 hours before counting. Another loopful from the same tube was taken at practically the same time and inoculated into tubes containing 10 cc. of plain broth and the tubes were

[2] We are greatly indebted to Dr. Martha Wollstein and to Dr. Louise Pearce who conducted the tests with the meningococcus and gonococcus, respectively. Their familiarity with the cultural conditions of the two microorganisms was of special value and service.

incubated for 48 hours. It was found that at a certain dilution, using the plating method, there was a very abrupt falling off in the number of colonies in the plates. This point was always marked in the broth tubes by clear-cut differences in appearance of the incubated tubes. The organisms in the lower dilution produced marked turbidity and in the next higher dilution remained absolutely clear. It was apparent that from 50 to 80 organisms were necessary to inoculate a 10 cc. broth tube, so that anything below this number would not show in this culture media. On the other hand, each organism in the plates produced a colony. Having found at the beginning of the work that this point of abrupt decrease in the number of colonies, using the plate method, came always at the same dilution indicated by no growth in the tubes, the plate method was no longer used on account of the large number of drugs tested.

In the streptococcus tests one or two drops of a 24 hour bouillon culture of an ordinary hemolytic strain of streptococcus were added to each dilution tube. After 3 hours at 20°C. a loopful was taken from the bottom of each tube and plated in plain agar or blood agar. A bacterial control was run in distilled water and plated immediately after mixing and also at the end of the incubation period. By such a control it was possible to estimate the percentage of killing when complete killing did not occur. The plates were incubated at 37°C. for 18 hours and the results recorded.

In the case of the meningococcus the tests were made by Dr. Wollstein. Average 24 hour growths of the microorganism on sheep serum agar were washed off with 2 cc. of sterile distilled water. 0.5 cc. of this well mixed suspension was added to each tube containing the compound dilution. This was allowed to stand for 3 hours at 20°C. Then 0.2 cc. of each tube was planted on sheep serum agar slants. These were incubated for 48 hours and the readings taken. Controls accompanied each experiment.

For the gonococcus tests conducted by Dr. Pearce an adult strain of the organism was employed. 24 hour growths on ascitic veal agar were washed off with 3 to 5 cc. of normal salt solution. The exact amount of salt solution depended upon the profuseness of the growth. The object was to obtain a decidedly cloudy but not milky suspension of the bacteria. 0.5 cc. of this suspension was then added to the tubes containing the substance dilutions and allowed to stand at

20°C. for 3 hours. 0.2 cc. was then pipetted from the bottom of the tubes and planted on an ascitic veal agar slant and incubated for 48 hours. The readings were then taken. Controls were run in each experiment.

In all the above experiments the tests were run in duplicate.

TABLE I.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetamide	1,600	800–1,600	800	800–1,600
Chloroacetmethylamide	1,600	800	3,200	1,600
Chloroacetethylamide	800	800–1,600	3,200	6,400
Chloroacetdimethylamide	1,600	1,600	800	1,600
Chloroacetdiethylamide	1,600	1,600	1,600	1,600
Chloroacetpiperidide	200	800		
β-iodopropionamide	+*	+	800	800

*+ indicates growth after exposure to a dilution of 1:200.

The results obtained with the hexamethylenetetramine quaternary salts prepared from chloroacetamide and the chloroacetyl derivatives of the simpler aliphatic amines will be found in Table I. With but few exceptions these substances were found to kill the organisms used for the test in dilutions of at least 1:1,600 in 3 hours at 20°C. On the whole, but little variation in action, at least of a magnitude which could be detected by the dilution scheme employed, was obtained by the addition of alkyl radicals to the amide nitrogen in the chloroacetamide salt. The exceptions presented by the behavior of the methyl- and ethylamide derivatives toward the meningococcus are worthy of note. With these substances the action was observed to be about four times as great as that of the unsubstituted chloroacetamide salt or its dimethyl derivative. The unusual activity of the compound obtained from chloroacetylethylamine against gonococcus is also noteworthy.

When it is considered that substances of purely aliphatic nature are represented in this series, the bactericidal power observed is quite unusual. Formaldehyde, which is considered one of the most powerful of the aliphatic bactericides, when tested by the same tech-

nique was found to kill *Bacillus typhosus* in a maximum dilution of 1: 1,200. In addition, the molecular weights of these substances are approximately ten times that of formaldehyde, so that if the comparison were strictly drawn the observed figures should be multiplied by ten. On this basis they far exceed formaldehyde in molecular bactericidal power. The activity of the substances of this group as bactericides is attributable entirely to the presence in them of the hexamethylenetetramine molecule.[3]

The comparative results obtained against *Bacillus typhosus* by the same technique with other aromatic substances which have been regarded in the past as strong organic antiseptics are given in Table II. Unfortunately the tests were restricted to *Bacillus typhosus*. Among the substances given in Table I and in those to follow, many will be found which are as active or more active than any given in this table.

TABLE II.

3 Hours at 20° C.

Substance.	*B. typhosus* killed in dilution of 1:
Formaldehyde	1,200
Phenol	+*
Lysol	400
Trichlorophenol	800
Tribromo-*p*-cresol	1,600
Tetrabromo-*o*-cresol	1,600
Tribromo-*m*-xylenol	1,600
Gentian violet	3,200

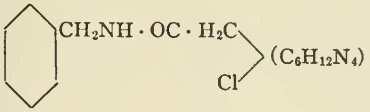
* + indicates growth after exposure to a dilution of 1:200.

In an attempt to determine what influence might result from the substitution of the halogenacetic acid radical by that of other halogen fatty acids, it was found that the higher α-halogen fatty acid

[3] There is evidence that the hexamethylenetetramine molecule does not always persist as such when its quaternary salts are dissolved in water, but undergoes a decomposition which yields methylene derivatives of the corresponding amines. The relation of these substances to the observed effects we shall make the subject of a subsequent communication.

derivatives failed to react with hexamethylenetetramine. The derivatives of β-iodopropionic acid, however, were found to react smoothly with the base to give quaternary salts. The ineffectiveness of the salt obtained from β-iodopropionamide in particular served to indicate the scant promise offered by the further employment of this acid and that the best results would be obtained by the continued use of the halogenacetyl group.

In the logical development of the above substances of purely aliphatic origin, the effect of the introduction of the aromatic nucleus into the alkyl group situated on the amide nitrogen was studied. The opportunity for this was furnished by the quaternary salts obtained from the chloroacetylbenzylamines possessing the following structural formula:



It was thought that here the usual antiseptic influence of the aromatic nucleus would appear, but, as will be seen from the results presented in Table III, this did not conform to the expectations. In interpreting the results, however, the greater molecular weights of these substances should not be neglected. Nevertheless, in those cases in which the observations fell below 1: 800 the chemical structure alone must be held responsible. Owing to the irregular character of the fluctuations observed it is difficult to deduce from this table any general relationships between the chemical constitution and the bactericidal power. In the case of the streptococcus and gonococcus, however, the introduction of the methyl group seemed to enhance the activity. The methoxy derivatives also appeared to be more effective than the corresponding acetoxy compounds. It is possible that a series of salts prepared from the mono- substituted benzylamines would have afforded a less confusing and more comparable group of substances for study. The difficulty of procuring such material and the pressure of other work were obstacles to the further extension of this chemical type.

On turning to the more easily accessible chloroacetylanilines, a series of substances was obtained which afforded ample opportunity

TABLE III.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylbenzylamine......	1,600*	800	1,600	800
Chloroacetyl-*o*-methylbenzylamine.....................	1,600*	3,200	1,600	3,200–6,400
p-acetaminoiodoacetylbenzylamine.....................	800*	200	1,600	800
1-methyl-4-acetaminochloroacetylbenzylamine..........	+†	800	800	1,600
1, 2-diacetoxychloroacetylbenzylamine...............	800*	800–1,600	800	400
1, 2-dimethoxychloroacetylbenzylamine...............	1,600*	800	400	800
1-acetamino-4-ethoxychloroacetylbenzylamine..........	800–1,600	800–1,600	400	800
β-acetoxy-*α*-naphthochloroacetylbenzylamine.............	800	1,600	800	800
β-methoxy-*α*-naphthochloroacetylbenzylamine..........	1,600	3,200	1,600	1,600
m-carbethoxychloroacetylbenzylamine..................	800	400	400	800
m-carbamidochloroacetylbenzylamine..................	+		3,200	1,600
Diethylaminoethyl ester of *m*-carboxychloroacetylbenzylamine.....................	200	800		

* Tests in these cases were made at 37° C.

† + indicates growth after exposure to a dilution of 1:200.

for ascertaining the influence of the introduction of groups into the benzene nucleus. These substances possessed the following structural formula, in which any group X may occur in the *ortho*, *meta*, or *para* positions:

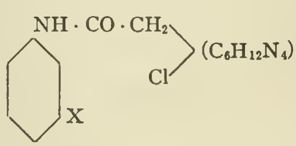


Table IV shows that the linkage of hexamethylenetetramine by means of the chloroacetyl radical with the simpler aromatic amines

TABLE IV.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylaniline..................	800	1,600	1,600	3,200
Chloroacetyl-*o*-toluidine.............	1,600*	1,600	1,600	1,600
Chloroacetyl-*p*- "	800	800	1,600	3,200
Chloroacetyl-*m*-4-xylidine............	800	1,600	1,600	3,200
Chloroacetyl-*ψ*-cumidine.............	800	1,600	3,200	3,200
Chloroacetyl-*α*-naphthylamine........	800	800	800	1,600–3,200
Chloroacetyl-*β*- "	1,600*	800	6,400	6,400
6-chloroacetylaminoquinoline	3,200	3,200	1,600	1,600
Chloroacetyl-*o*-chloroaniline..........	1,600	1,600	800	3,200
Chloroacetyl-*p*-bromoaniline..........	1,600	1,600	1,600	3,200
Chloroacetyl-5-iodo-*o*-toluidine.......	1,600	800	1,600	3,200
Chloroacetyl-*m*-nitraniline............	3,200	3,200	3,200	3,200
Chloroacetyl-*m*-nitro-*p*-toluidine......	800			
o-chloroacetylaminophenol	1,600	800	800	3,200–6,400
Chloroacetyl-*o*-anisidine.............	800		3,200	1,600
Chloroacetyl-*p*- "	3,200	1,600		
β-iodopropionyl-*o*- "	+†	200	400	400
Chloroacetylmethylaniline		1,600	800	1,600
Chloroacetyldiphenylamine...........	400	200	1,600	1,600
p-chloroacetylaminobenzoic ethyl ester	1,600	3,200	1,600	3,200
Chloroacetylnovocaine...............			1,600	3,200–6,400
o-chloroacetylaminobenzyl alcohol	800	800–1,600	800	800
o- " benzoate...	800	3,200	1,600	
o-chloroacetylaminophenyl " ...	200	1,600	800	3,200
o- " *p*-nitro-benzoate........................	800	1,600–3,200	1,600	1,600
m-chloroacetylaminoacetophenone....		1,600–3,200	800	1,600

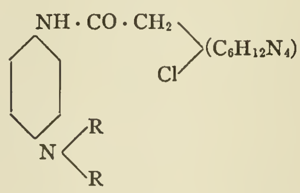
* Tests were made at 37° C.

† + indicates growth after exposure to a dilution of 1:200.

produced a group of substances possessing definite bactericidal properties. Contrary to the results obtained with the substituted benzyl compounds described in the previous communication, it was found that alterations in the benzene nucleus by the usual substituents did not result in sharp differences in the bactericidal effect, at least of a magnitude which could be revealed by the scheme of dilutions employed. Many instances are to be found, however, in which the ac-

tivity of the simplest member, the salt obtained from chloroacetylaniline, has been definitely improved. Among these may be mentioned the substances obtained by the introduction of the methyl, chlorine, bromine, iodine, and nitro groups. Such chemical variations, however, did not always result in an improvement. In many cases the bacteria were killed in dilutions of 1: 3,200 or even 1: 6,400. On the whole, of the microorganisms employed, the gonococcus was the least resistant towards the members of this group. In the absence of indications of a more decided character or of greater regularity there was little assurance of obtaining more powerful bactericides by the further use of these substituents.

The results, however, assumed a different character by the adoption of a new type of variation within this group of substances. This consisted in the use of the dialkylamino group as a substituent in the nucleus of the parent chloroacetylaniline quaternary salt. These substances were prepared by the reaction of the chloroacetylaminodialkyl anilines with hexamethylenetetramine and possessed the following structure:



in which R may be methyl, ethyl, etc. The bactericidal results are given in Table V.

TABLE V.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
p-chloroacetylaminodimethylaniline	+†	800–1,600	1,600	1,600
p-chloroacetylaminodiethylaniline........		3,200–6,400	1,600	3,200
p-chloroacetylaminodipropylaniline*		6,400	3,200	6,400
p-chloroacetylaminobenzylethylaniline* ...		6,400	6,400	12,800
m-chloroacetylaminodimethylaniline		1,600	400	400

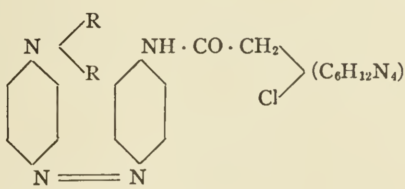
* In these cases, owing to the sparing solubility in water, one mol. of N HCl was used to dissolve the substances.

† + indicates growth after exposure to a dilution of 1:200.

Our attention was first called to the desirability of developing this series by the apparent partial specificity of the dimethyl compound for the streptococcus as compared with the effect observed upon *Bacillus typhosus.* Later its effectiveness against the meningococcus and gonococcus was found to be equally marked. The later members of the group were obtained by replacing the methyl groups by ethyl, propyl, and benzyl. In these tests *Bacillus typhosus* was unfortunately omitted. We are therefore in no position to state whether this organism is more resistant to these substances as a class.

The regularity of the response to this particular chemical alteration is strikingly shown by these results. A progressive improvement occurred in the bactericidal action upon all three species of bacteria upon proceeding from the dimethyl to the diethyl, dipropyl, and finally the benzylethyl derivatives, and this in spite of the constant increase in molecular weight. If the increase with each dilution had not been so great, it is probable that all the columns of the table would have shown the regularity of the gonococcus results. The striking feature of the observations is the magnitude of the effect produced by such slight alterations in a complicated molecule. The inferior results obtained with the *meta*-dimethylamino compound, the last in the table, would indicate that the relative positions occupied by the substituents in the nucleus are modifying factors.

The efficacy of these groups was still further demonstrated by their use in another class of substances obtained by the addition of hexamethylenetetramine to chloroacetylaminoazobenzene derivatives, in which the base was attached by the chloroacetylamino side-chain to one nucleus and the dialkylamino group to the other as presented in the following formula:



In Table VI the salt obtained from chloroacetylaminoazotoluene, which contains no dialkylamino group, is first given as an

TABLE VI.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
p-chloroacetylaminoazotoluene	3,200	3,200	3,200	1,600
p-chloroacetylaminobenzeneazo-*p*′-dimethylaniline*	800	12,800	3,200	3,200
p-chloroacetylaminobenzeneazo-*p*′-diethylaniline	+‡	12,800	1,600	800
p-chloroacetylaminobenzeneazo-*p*′-dipropylaniline*		12,800	800	800
p-chloroacetylaminobenzeneazo-*p*′-benzylethylaniline*		12,800	1,600	800
Benzeneazo-*m*-chloroacetylaminophenol†		3,200		

* In these cases 1 mol. of N HCl was employed to dissolve the substance.

† Solution made by the use of 1 mol. N NaOH.

‡ + indicates growth after exposure to a dilution of 1:200.

object for comparison.[4] The action of this substance upon the different species of bacteria was fairly uniform. The introduction, however, of the dimethylamino group into that position in the molecule farthest removed from the location of the hexamethylenetetramine nucleus resulted in a marked difference. But slight alteration, if any, was observed in the meningococcus, a slight improvement towards the gonococcus, and a considerable reduction in the action upon *Bacillus typhosus*. With the streptococcus, however, the change was profound. The action was increased at least fourfold. The efficacy of this type of chemical modification against the streptococcus was still further confirmed by the replacement of the dimethyl group by the diethyl, dipropyl, and benzylethyl groups. These variations produced compounds which, in spite of the increased molecular weight, exhibited a degree of action of the same order. On the other hand, when tested against the other organisms they were found to be bactericidally less active than the dimethyl compound. We have here an interesting instance of specificity for streptococcus.

[4] The simpler chloroacetylaminoazobenzene derivative could not be conveniently used because of its sparing solubility in water.

Here again the hexamethylenetetramine molecule was found to be a factor in bringing out this effect. This was directly demonstrated in the following manner: *p*-aminobenzeneazodiethylaniline may be considered as the third substance mentioned in the table deprived of hexamethylenetetramine and the $-CH_2CO-$ radical which serves only as a connecting link. This dye was found to kill the streptococcus in a maximum dilution of 1:3,200, an effect which, though marked, is still but one-fourth of the result obtained with its hexamethylenetetraminium salt.

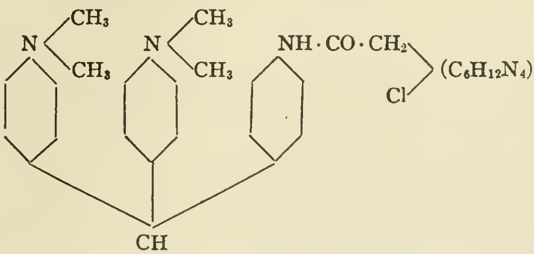
TABLE VII.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
p-chloroacetylamino-*p'*,*p''*-tetramethyldiaminotriphenylmethane (*p*-chloroacetylaminoleukomalachite green)*	400	12,800	200	200
o-chloroacetylamino-*p'*,*p''*-tetraethyldiaminotriphenylmethane*..............		12,800	6,400	1,600
p-chloroacetylamino-*p'*,*p''*-tetraethyldiaminotriphenylmethane*..............		51,200	1,600	1,600
Chloroacetyltriphenylamine.............		3,200-6,400	800	800

* In these cases 1 mol. of N HCl was employed to dissolve the substance.

The group of substances given in Table VII headed by the hexamethylenetetramine quaternary salt of chloroacetyl-*p*-aminoleukomalachite green,



afforded the opportunity of still further testing the value of dialkylamino compounds against the streptococcus. This salt, as well as its homologs, displayed a marked specificity for this microorganism, kill-

ing it in a dilution of at least 1: 12,800, whereas the effect on the other forms was relatively weak. In the next two compounds the methyl groups were changed to ethyl groups, and in one case the chloroacetyl-amino radical was shifted to the *ortho* position. The dilution of 1: 12,800 was usually the highest dilution employed for the tests in the routine procedure, but fortunately the experiments performed with the third substance were carried further. This preparation was found to kill the streptococcus even in a dilution of 1: 51,200, making it probable that the first and second substances also would have been found to kill above 1: 12,800.

That here also the hexamethylenetetramine molecule is an essential factor was proven as follows. The first substance given in the table when deprived of this base and its connecting group is nothing else than *p*-aminoleukomalachite green. This substance required a concentration of at least 1: 800 to kill the streptococcus in 3 hours. In other words, the hexamethylenetetraminium salt derived from it was at least sixteen times more active.

In the course of the present work our interest was centered for a time in the study of the biological properties of the hexamethylenetetramine quaternary salts obtained from the chloroacetylalcamines. Our attention was attracted first to this group of substances by the powerful bactericidal properties of the simplest representative, that obtained by the addition of hexamethylenetetramine to chloroacetylaminomethanol.[5]

$$\left.\begin{matrix} HO\cdot CH_2\cdot NH\cdot CO\cdot CH_2 \\ Cl \end{matrix}\right\rangle (C_6H_{12}N_4)$$

This substance, which possesses the above structural formula, is the first given in Table VIII. It is seen to possess a marked action against all the species used with the exception of the streptococcus. Because of the unusual effectiveness of this product it was hoped that its suitable chemical variation might lead to a series of very active substances.

[5] This substance was first prepared by Einhorn and Göttler (*Ann. d. Chem.*, 1908, ccclxi, 150), who also recognized its antiseptic properties.

TABLE VIII.

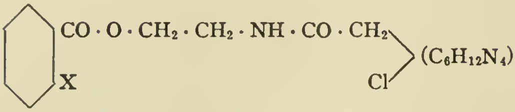
3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylaminomethanol	3,200	400–800	6,400	6,400
Iodoacetylaminoethanol		400	1,600	800
Chloroacetylaminoisopropanol		200		
δ-chloroacetylamino-n-butanol		400		
β-chloroacetylamino-γ- "		800		
γ-chloroacetylamino-β-methyl-β-butanol		400		
α-phenyl-α-oxy-β-chloroacetylaminoethane		1,600		1,600
β-phenyl-β-oxy-α-chloroacetylaminopropane		800		
Chloroacetylaminoethyl ether		1,600		
Chloroacetyl-o-methylphenoxyethylamine		800		

The chemical development of this substance was attempted in two ways: first, by the replacement of its methanol group by the ethanol, propanol, butanol, etc., radicals; and second, by the acylation of the methanol hydroxyl group with various acid radicals. Inthe latter scheme, however, chemical difficulties were encountered which compelled the use of its homologs, in particular the ethanol derivative, as the basis for the study of the effect of acylation.

Table VIII presents the behavior principally towards streptococcus of the substances obtained by the first method of chemical variation. The results show that the first member of the group, the salt obtained from chloroacetylaminomethanol, is the most powerful, so that no bactericidal increase was to be gained by such a chemical procedure.

Quite a different result was obtained by the use of the second scheme of chemical variation, as will be seen in Table IX. Unfortunately the inaccessibility of the acylated methanol derivatives made impossible a direct comparison of the effect of acylation upon the chloroacetylaminomethanol salt itself. The results must therefore be referred to the parent unacylated alcamine compound in question for a strict comparison. The structural formula of this group of substances may be represented as follows, X being any substituting group:



A glance at the table will show that we have here another group of hexamethylenetetraminium salts with strong bactericidal properties surpassing in this respect the parent chloroacetylalcamine compound

TABLE IX.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylaminoethyl benzoate	800	400	1,600–3,200
" *o*-methylbenzoate	1,600	3,200	1,600
" *p*- "	3,200	1,600	800
" β-naphthoate	3,200	6,400	6,400
" *o*-nitrobenzoate	800	3,200	1,600
" *p*- "	6,400	6,400	3,200
" *p*-methoxybenzoate	3,200		
" acetylsalicylate	800		
" *p*-diethylaminobenzeneazo-*p'*-carboxylate	6,400–12,800	400	400
Chloroacetylamino-γ-propyl *p*-nitrobenzoate	1,600		
" -γ- " *p*-methoxybenzoate	1,600–3,200	800	1,600–3,200
Chloroacetylethylaminoethyl *p*-nitrobenzoate	3,200		
Chloroacetylphenylaminoethyl *p*- "	800		3,200–6,400

By the introduction of the simplest aromatic acid, benzoic acid, the bactericidal power of the parent iodoacetylaminoethanol salt was doubled, except for the meningococcus. The use of the substituted benzoic acids, such as the methyl, nitro, and methoxybenzoic, and naphthoic acids, in most cases still further improved the action. In the case of the nitrobenzoates the *para* compound seemed more effective than its *ortho* isomer. With the methylbenzoyl derivatives the *para* compound was also more active towards the streptococcus than its *ortho* isomer. With meningococcus and gonococcus the reverse was the case. The specificity of the *p*-diethylaminobenzeneazo-*p'*-carboxylate for streptococcus was to be expected from the results already discussed in connection with Table VI. This substance possesses the

diethylamino group. With the few acids studied, the best results were obtained with the *p*-nitrobenzoyl and *β*-naphthoyl compounds. The results yielded by the use of other alcamines, such as aminopropanol, ethylaminoethanol, etc., would seem to indicate that the optimum effect is to be obtained with the aminoethanol series.

In this group of substances but a few representatives were made and tested. By the use of numerous other acids a much broader series might be developed for study with the possibility of obtaining more active preparations. However, the observations obtained with this small group of substances serve to demonstrate again to what extent the bactericidal effect may be altered by relatively small changes in the molecule. Here, as in the case of the benzylhexamethylenetetraminium salts discussed in the previous communication, the degree of action is determined by the character and position of the substituents in the benzene nucleus. The main source of the bactericidal effect, however, is still the hexamethylenetetramine molecule.

TABLE X.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylurea	3,200	800–1,600	1,600	1,600
α-chloroacetyl-β-methylurea	800	400–800	1,600	1,600
α-chloroacetyl-β-benzylurea	400	400	800	1,600
Chloroacetylurethane	400		1,600	800

Still another type of hexamethylenetetramine quaternary salt included in the investigations was that represented by the compound obtained by the reaction of chloroacetylurea with hexamethylenetetramine. In Table X it is seen that for a purely aliphatic substance it exhibited a strong bactericidal power. It was hoped that by turning to the substituted ureas this action might be improved. The experience with the methyl and benzyl compounds, however, showed only a diminution of the activity.

Up to this point the substances which have been the subject of discussion were all quaternary salts obtained from halogenacetylamino compounds. Two other types of substances were included in the

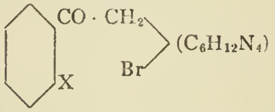
TABLE XI.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetone	1,600*			
ω-bromoacetophenone	1,600*	3,200	1,600	1,600
p-methyl-ω-bromoacetophenone	1,600	800	800	1,600
p-ethyl-ω- "	800			
1, 2-dimethyl-ω- "	3,200	3,200	3,200	6,400
1, 3-dimethyl-ω- "	1,600		800	1,600
m-nitro-ω- "		1,600		
p-methoxy-ω- "	800	800	3,200	6,400
p-ethoxy-ω- "	800	1,600	1,600	1,600–3,200
p-acetamino-ω- "	800	3,200	1,600	3,200
3-acetamino-4-methyl-ω- "			800	800
3-acetamino-4-tolyl ω-iodoethyl ketone	800	1,600–3,200	12,800	12,800
1, 2-diacetoxy-ω-iodoacetophenone	400	1,600	800	800
β-[ω-bromoaceto]-quinaldine	200	3,200	3,200	3,200

* Tests were made at 37° C.

study in which hexamethylenetetramine was joined by means of the halogenacetyl group first to hydrocarbons and then to alcohols. The first of these groups, which was prepared by the addition of halogen ketones to hexamethylenetetramine, may be represented by the following formula:



The results of the experiments made with these substances are contained in Table XI. The bactericidogenic property of hexamethylenetetramine was again demonstrated. The first member, the salt obtained from chloroacetone, was found to kill *Bacillus typhosus* in a dilution of 1: 1,600, which is again striking for an aliphatic substance. Among the aromatic representatives the majority killed one or another of the species tested in dilutions of 1: 1,600 or more. The behavior of the 1,2-dimethyl-ω-bromoacetophenone and *p*-methoxy-ω-bromoacetophenone derivatives toward the gonococcus and the action of the salt obtained from 3-acetamino-4-tolyl ω-iodoethyl

ketone on the gonococcus and the meningococcus are worthy of note. It is seen that the chemical constitution of the compounds determines in a degree the bactericidal power, but any definite regularity is far from apparent. As in many instances to be seen in the other tables, the result of a particular chemical variation upon the bactericidal power varies according to the organism used for the test. An interesting instance of the influence of the relative positions occupied by substituents in the benzene nucleus upon the bactericidal effect is shown by the differing action of the 1,2- and the 1,3-dimethyl-ω-bromoacetophenone salts towards the meningococcus and the gonococcus. The former substance is four times more active than the latter.

The results yielded by the salts obtained from halogenacetyl esters are given in Table XII. The ease of saponification of this chemical

TABLE XII.

3 Hours at 20° C.

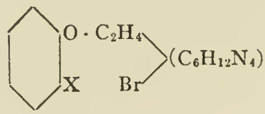
Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Ethyl bromoacetate	400	1,600	800	800
Phenyl "	800	3,200	1,600	3,200
Bornyl "	3,200			
Menthyl "	800	1,600–3,200	1,600	1,600
Ethyl β-iodopropionate	+*	200	200	400
Anisoylglycol chloroacetate	1,600	1,600	1,600	1,600
p-nitrobenzoylaminoisopropyl chloroacetate	1,600	1,600		1,600

*+ indicates growth after exposure to a dilution of 1:200.

type limited its more extended development. The table demonstrates the bactericidogenic properties of hexamethylenetetramine in this combination also. The relatively low bactericidal power of the β-iodopropionyl derivative is also in line with the results obtained with other derivatives of this acid.

In the course of the work still other connecting groups than the halogenacetyl radical were used in order to combine hexamethylenetetramine in the form of quaternary salts with other molecular group-

ings. Bromoethyl alcohol by virtue of its alcoholic hydroxyl group may combine with acids to form bromoethyl esters or may be considered the mother-substance of the bromoethyl ethers. These bromoethyl derivatives react readily with hexamethylenetetramine, giving the two following classes of salts:



$$\begin{matrix} RCO \cdot O \cdot C_2H_4 \\ Br \end{matrix} \Big> (C_6H_{12}N_4)$$

The results obtained with the first of these, the bromoethyl ether salts, are given in Table XIII. It is to be observed that this type was, on the whole, most active against the meningococcus and the

TABLE XIII.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Phenyl bromoethyl ether............	200	+	400	800
o-methylphenyl bromoethyl ether	+*	400	1,600	1,600–3,200
m- " " "	400		1,600	1,600
p- " " "	400		400	800
α-naphthyl bromoethyl ether.........	+	800	3,200	12,800
β- " " "	800	1,600–3,200	1,600	3,200–6,400
p-bromophenyl " "	200	+	3,200	1,600
Tribromo-*p*-cresyl " "		3,200	800	800
o-acetaminophenyl " "			400	400
p- " " "			200	200

*+ indicates growth after exposure to a dilution of 1:200.

gonococcus. The partial specificity of the α- and β-naphthol bromoethyl ether salts for the gonococcus is especially noteworthy. The α-compound, which killed the gonococcus in a dilution of 1:12,800, was ineffective against *Bacillus typhosus* in a concentration of 1: 200. These instances, together with the other substances mentioned in the table which were found to kill one or the other microorganism in dilutions of 1: 1,600 or 1: 3,200, still further indicate how general in character is the bactericidogenic property of the hexamethylenetetramine molecule

TABLE XIV.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Bromoethyl acetate........................	+*		800	400
" benzoate........................	200	400	400	800
" *p*-nitrobenzoate................	+		800	800
Bromoethylphthalimide....................	+	+	400	800

* + indicates growth after exposure to a dilution of 1:200.

In the case of the bromoethyl ester salts (Table XIV) the introduction of the hexamethylenetetramine molecule is seen to be considerably less effective. However, here again the best results were obtained with the meningococcus and the gonococcus. The last substance in the table is not an ester but a bromoethylamino compound. This also was most active against the meningococcus and gonococcus. It would seem from both this series and the previous group of substances that there is something in the chemical nature of the salts obtained from bromoethyl compounds which renders them most active against these two species of bacteria. Although not as marked, this may be considered analogous to the specific effect of the dialkylamino group upon the streptococcus.

It is highly probable that the further development of any of the leads which have been indicated in these papers might eventually furnish more active preparations which would be of chemotherapeutic value.

In conclusion we wish to present the results obtained in a few experiments on the effect of serum and protein on the bactericidal action of several of the compounds mentioned in the preceding tables. It so happened that in these serum-compatibility tests the technique employed was that described in the preceding paper. For this reason the parallel observations made with solutions of the compounds in physiological salt solution are a dilution higher than those to be found in the preceding tables. Table XV presents the results of these tests.

TABLE XV.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in 4 hrs. at 37° in a dilution of 1:	
	In physiological salt solution.	In horse serum.
Chloroacetyl-*o*-toluidine	3,200	3,200
Chloroacetylaminomethanol	6,400	3,200
ω-bromoacetophenone	1,600	800
1, 2-diacetoxychloroacetylbenzylamine	1,600	800

It is seen that in the case of the salt obtained from chloroacetyl-*o*-toluidine the action was not inhibited by serum. In the other cases the observed effect was reduced by half in the presence of serum. It is possible that in these cases the apparent inhibition was accentuated by the dilution scheme employed, and that in reality but little relative inhibition occurred. With a few other compounds of this class tested by a different technique a much greater relative inhibition of the bactericidal action was observed. From these experiments we may at any rate conclude that the bactericidogenic hexamethylenetetramine portion of the molecule does not in itself cause serum-incompatibility. The source of this must be sought in the remainder of the molecule.

TABLE XVI.

Hexamethylenetetramine quaternary salt of	Gonococcus killed in 2 hrs. at 20° in a dilution of 1:	
	In aqueous solution.	In 5 per cent sodium caseinate solution.
Chloroacetyl-β-naphthylamine	3,200	1,600
p-methoxy-ω-bromoacetophenone	6,400	6,400
Chloroacetylnovocaine	6,400	6,400
α-naphthyl bromoethyl ether	12,800	6,400
Choloroacetylaminoethyl-*p*-nitrobenzoate	3,200	3,200
3-acetamino-4-tolyl ω-iodoethyl ketone	12.800	1,600

In Table XVI will be found the results of a series of tests in which the substances were dissolved in a 5 per cent sodium caseinate solution. The gonococcus was here used and the technique employed was the same as that described in the other gonococcus tests. In only one

case, the last given in the table, was any marked inhibition to be observed. With the remaining substances mentioned relatively little or no inhibition was observed.

SUMMARY.

The extension of the study of the quaternary salts of hexamethylenetetramine to those obtained by the addition of this base to the most varied types of substances containing aliphatically bound halogen has demonstrated that the introduction of the hexamethylenetetramine nucleus in this manner results in the production of bactericidal substances or enhances the bactericidal action if already present.

In particular it was found possible by the use of the halogenacetyl group, XCH_2CO, as a connecting link, to furnish primary and secondary aliphatic and aromatic amines, alcohols, and hydrocarbons of the most varied character with the hexamethylenetetramine molecule and to study the relation between chemical constitution and bactericidal action in the series of substances so prepared. Because of the variety of chemical types studied, the results are too involved for a detailed summary here.

Many of the substances were found to be very powerful bactericides, and in a number of instances derivatives of purely aliphatic nature were found to possess an unusual bactericidal power.

Bacillus typhosus, streptococcus, meningococcus, and gonococcus were the microorganisms used for the tests, and striking instances of partial specificity were observed. This specificity was found to favor not one species alone, but instances were found in which each of the types of bacilli was shown to be especially susceptible to one or another of the particular types of compound employed. The source of this partial specificity is to be sought not in the hexamethylenetetramine nucleus itself but in the molecule to which it is attached.

The action of some of the substances was tested in the presence of serum or protein and was found to be not at all or only slightly inhibited. In other cases marked inhibition occurred. The factors controlling the serum- or protein-compatibility of these substances are likewise to be sought in that portion of the molecule other than the hexamethylenetetramine.

THE PROTECTION OF PATHOGENIC MICROORGANISMS BY LIVING TISSUE CELLS.

By PEYTON ROUS, M.D., and F. S. JONES, V.M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plate 90.

(Received for publication, February 10, 1916.)

The discovery by Metchnikoff of the purposeful character of phagocytosis has so stimulated investigation of the defensive activities of tissue cells that phagocytosis and bacterial destruction are at present almost synonymous in the general mind. The old view that leukocytes provide ingested organisms with a culture medium and a means of transport is now mentioned only in historical résumés. It is known that bacteria may be ingested alive—Metchnikoff himself utilizes this fact in his demonstration of the importance of cells for immunity[1] —and it is known also that a cell may take up too many microorganisms and dying of a surfeit, as one might say, may fail to kill them. But such occurrences are regarded as mere incidents in the process of destruction. The possibility that in certain instances cells not only fail to kill the organisms they ingest, but actively protect them from circulating antibodies seems not to have been considered. Yet the question thus raised has more than passing interest. There are a number of important diseases, among them leprosy, tuberculosis, gonorrhea, Leishmania, caused by microbic parasites which live more or less habitually within tissue cells. The part played by the host cells in the life of such microorganisms and also in the distribution within the body of the diseases they induce has obvious importance.

Unfortunately it is not possible to make direct *in vitro* tests with the microorganisms mentioned and the cells in which they live, for the reason that they fail to give rise to circulating antibodies active

[1] Metchnikoff, E., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 679.

enough to be suitable for the tests. But the problem can be approached by means of artificial systems. It is possible, for example, to submit leukocytes that have ingested bacteria to a bactericidal serum and observe its effect on the intracellular organisms.

Protection against a Foreign Antiserum.

In a first experiment we have used *Bacillus typhosus*, the leukocytes of the guinea pig, which, according to Pettersson,[2] contain no substances destructive to typhoid bacilli, and the serum of normal rabbits, which is strongly bactericidal for the organism.

Experiment 1.—Washed leukocytes from two sterile, 18 hour, aleuronat exudates of the guinea pig's peritoneal cavity were made into a single thick suspension with Locke's solution, and incubated with typhoid bacilli in the presence of a much diluted mixture of fresh guinea pig serum and antityphoid rabbit serum. The smallest amount of the mixture that would ensure good phagocytosis had been previously determined. Twice this amount was employed. The suspension of typhoid bacilli consisted of four 24 hour slant agar cultures of different strains[3] made up in 80 cc. of Locke's solution.

After 1 hour's incubation films from the phagocytic mixture and from a control mixture without serum were examined, with Manson's stain. Only in the former was phagocytosis observed. It was profuse, though there were still many free bacteria. Now small portions of the mixtures and of other control mixtures were added to large amounts of fresh, normal rabbit serum, and the incubation was continued 2 hours longer. Plating was then done in equal portions of agar. Duplicate tests were made throughout. For the dilutions Locke's solution was used.

As the experiment shows, leukocytes can protect ingested bacilli from the action of a bactericidal antiserum.

The mixture of leukocytes and bacteria subjected to preliminary incubation without serum (Mixture 2), and consequently free of phagocytosis at the end of the first hour, gave many more colonies in the plates made 2 hours after the addition of rabbit serum than did Mixtures 3 and 4 from which leukocytes were absent. This was probably due to the protection of ingested organisms, despite the fact

[2] Pettersson, A., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1905, xxxix, 423.

[3] These were laboratory strains known respectively as Board of Health, Metchnikoff, Wassermann, and New York Hospital, which had been under cultivation for more than 2 years.

TABLE I.

Mixture.	Locke's solution.	Leukocytes.	Bacteria.	50 % guinea pig serum.	2 % antityphoid rabbit serum.	A.	Colonies per square cm. after 17 hrs.	B.	Colonies per square cm. after 17 hrs.
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	1 hr.'s incubation 0.3 cc. from each tube was mixed with 2.4 cc. normal rabbit serum; and after 2 hrs.' more incubation 0.5 cc. of this mixture was plated in 6 cc. agar.		Same as A except that 0.3 cc. was mixed with 2.4 cc. Locke's solution instead of serum.	
1	—	0.2	0.2	0.1	0.1		211 294		About 500.
2	0.2	0.2	0.2	—	—		72 49		Innumerable.
3	0.2	—	0.2	0.1	0.1		10 13		Exceedingly numerous.
4	0.4	—	0.2	—	—		25 27		Innumerable.

that the bacteria were all outside the cells at the time the rabbit serum was added. For the rabbit serum itself was able to cause phagocytosis and did actually cause this in the mixture, as the films show. Presumably it brought about the ingestion of some living bacteria which were then protected from its further action by the cells containing them.

The character of the protection was not determined in this experiment. The results of Table I can be interpreted otherwise than wholly in terms of bactericidal action. The serum was agglutinative; and agglutination can of itself produce a reduction in the number of colonies from a bacterial suspension. The leukocytes might have protected the bacilli merely mechanically against clumping and thus have brought about the results seen in the plates. Obviously, for further work a non-agglutinating bactericidal agent was desirable.

An Indicator of Cell Death.

The question came up, furthermore, whether the leukocytes exerting a protective influence were alive. Rabbit serum contains an hemolysin for guinea pig erythrocytes, and might well kill the white cells of this species. To solve the point resort was had to tests with

trypan-blue. Evans and Winternitz[4] state that the dye rapidly colors the nuclei of dead cells but does not stain living ones. The following experiment confirms their observation.

Experiment 2.—A 4 day aleuronat exudate from the pleural cavity of a dog was washed and suspended in Locke's solution. It contained many large mononuclear cells capable of phagocyting rat erythrocytes. A part of the suspension was mixed with rat erythrocytes and dog serum, and incubated for 1 hour, after which an equal bulk of a freshly prepared and filtered solution of trypan-blue (0.02 gm. in 2.0 cc. of Locke's solution) was added and the cells forthwith examined. The nuclei of the majority of them failed to stain. None of the many cells that had phagocyted erythrocytes showed nuclear staining.

Other portions of the original suspension were kept in the ice box for several days and then treated in the same way. Most of the cells now failed to take up the rat corpuscles, and most stained promptly with trypan-blue. Among the few which did not stain were those which had just phagocyted rat cells.

The results of this experiment have been borne out by many subsequent observations involving injury to cells of other types, among them the cells liberated from tissue cultures by digestion of the plasma clot with trypsin.[5] Trypan-blue is a prompt and reliable indicator of whether cells are alive or dead.

Protection against an Inorganic Disinfectant.

The test with trypan-blue showed that rabbit serum is injurious to guinea pig leukocytes subjected to it under the conditions of Experiment 1. At the end of 2 hours' incubation about half the leukocytes were dead, as shown by the nuclear staining, whereas in control specimens incubated in salt solution, they were nearly all alive, very few stained cells being observed. Because of the unfitness of rabbit serum for our work, as thus manifested, it was necessary to find another bactericidal agent, one that would not harm the leukocytes or agglutinate the bacteria. Potassium cyanide proved to have both these qualities.

Clowes[6] was the first to demonstrate the difference in resistance of tissue cells and bacteria to potassium cyanide. As far as we are

[4] Evans, H. M., and Winternitz, M. C., unpublished work, cited by Evans, H. M., and Schulemann, W., *Science*, 1914, xxxix, 443.

[5] Rous, P., and Jones, F. S., *Jour. Exper. Med.*, 1916, xxiii, 549.

[6] Clowes, G. H. A., *Brit. Med. Jour.*, 1906, ii, 1548.

aware, his important observations have not been followed up. He found that tumor cells treated *in vitro* with cyanide in a concentration that killed bacteria remained capable of causing tumors on implantation. Our tests have shown that $\frac{N}{150}$ potassium cyanide in Locke's solution will destroy typhoid bacilli in heavy suspension while failing to kill guinea pig leukocytes, as shown by the trypan-blue test. Furthermore, the bacilli are not agglutinated. In Experiment 3 advantage has been taken of these facts.

Experiment 3.—This experiment closely followed Experiment 1 except that potassium cyanide was substituted for rabbit serum. A watery solution of potassium cyanide, isotonic with 0.95 per cent sodium chloride, was used, diluted with Locke's solution to $\frac{N}{100}$ concentration.

No phagocytosis was observed in Tube 2 after the preliminary incubation; but it was pronounced in Tube 1, though large numbers of bacilli were still free. The leukocytes were tested with trypan-blue at the time of plating and were found to be, in general, still alive, as proved by the fact that their nuclei failed to stain. In more concentrated potassium cyanide solutions they died early as shown by the stain. Some potassium cyanide was carried over into the agar plates but there it was greatly diluted. Control tests with regard to this point showed that the addition to agar of more than ten times the amount of potassium cyanide present in our plates failed to prevent or even to delay the growth of typhoid organisms.

TABLE II.

Mixture.	Locke's solution.	Leukocytes.	Bacteria.	50 % guinea pig serum.	2 % antityphoid rabbit serum.	A.	Colonies per square cm. after 17 hrs.	B.	Colonies per square cm. after 17 hrs.
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	1 hr.'s incubation then 1.2 cc. $\frac{N}{100}$ potassium cyanide added; 2 hrs.' more incubation and 0.2 cc. plated with 6.0 cc. agar.		Same as A except that 1.2 cc. Locke's solution added instead of the potassium cyanide.	
1	—	0.2	0.2	0.1	0.1		144 142		About 1,500
2	0.2	0.2	0.2	—	—		2 2		Innumerable.
3	0.2	—	0.2	0.1	0.1		0 0		About 1,500
4	0.4	—	0.2	—	—		0 0		Innumerable.

Here there was a marked protection of bacteria by the tissue cells containing them (Table II). The potassium cyanide entirely sterilized the suspensions in which the bacilli were free, whereas in the mixtures in which phagocytosis had occurred, many bacilli survived its action and grew in the plates. One or two colonies developed from Mixture 2, in which there was, supposedly, no phagocytosis because of the absence of serum. But, as is well known, phagocytosis takes place to a slight extent in the absence of serum. The result is readily explained on this basis.

Despite the results of this experiment, the evidence for a protection exerted by living cells specifically is not conclusive. For had the cyanide killed but one phagocyte in every several hundred,—and this may well have happened,—the gross number would be sufficient to permit of an interpretation of the results in terms of a protection exerted by dead phagocytes, not by living ones.

Protection against an Homologous Antiserum.

A second objection to experiments such as the foregoing is that they have little in common with the conditions in the animal body. We have sought to meet both these difficulties by employing red cells as the test object, placing phagocytes which contain them in an hemolytic antiserum derived from an animal of the sort furnishing the phagocytes. With such material it is easy to follow the fate of the ingested corpuscles and to determine by means of the trypan-blue stain whether the individual phagocytes exerting protection are alive or dead. And if the time element be disregarded, one can with good reason liken the conditions as regards the ingested rat corpuscles to those affecting pathogenic microorganisms existing within cells bathed with a lymph containing antibodies.

We have employed rat red cells allowing large mononuclear cells from an exudate in the dog's pleural cavity to phagocyte them, after which an anti-rat dog serum has been added to the mixture. In order to follow the fate of the ingested red cells it has been necessary to know what changes they would undergo when injured within the phagocytes by the hemolytic serum. For the corpuscles cannot lake as they would when free. Fortunately these changes proved easily

recognizable when several red cells were present side by side in a single phagocyte. They have been described and figured by Levaditi[7] and others, who noted them, as we have done, within phagocytes that had taken red cells out of an hemolytic mixture after the hemolysin had attached itself. Under such circumstances hemolysis goes on within the phagocyte with the result that the included corpuscles melt together, as it were, forming one or more large, orange-yellow hemoglobin-containing globules, which on pressure escape from the cell to dissolve instantly in the surrounding fluid. The contrast between these intracellular globules and red cells that remain intact after ingestion is pronounced (Fig. 1).

Experiment 4.—The phagocytes used were mononuclear cells of a 4 day aleuronat exudate in the pleural cavity of a dog. The exudate was washed twice and made into a thick suspension. It contained a very few red cells. The opsonizing serum was derived from the same dog, and so too the 25 per cent suspension of washed dog cells needed for the controls. The serum hemolytic for rat cells came from another dog which received three intravenous injections of rat erythrocytes on successive days and was bled 8 days thereafter. Preliminary examination of the two dogs' blood had shown that they did not agglutinate or hemolyze each other.

The form of the experiment was simple. Rat cells, dog exudate, and a little normal dog serum were mixed together, and, when phagocytosis had taken place, anti-rat dog serum was added to some portions of the mixture, and to others an equal quantity of Locke's solution. Incubation was resumed and from time to time the phagocyted red cells were observed for evidence of destruction. Two preliminary tests were necessary.

(*A*) A determination of the least amount of dog serum which would incite to phagocytosis. The dog serum used for its opsonins contained, as is usual, an hemolysin for rat cells. But the test showed that it could be used to incite phagocytosis in an amount far below the one producing visible erythrocytic change.

(*B*) A test to find out how much anti-rat dog serum was required to hemolyze free rat cells so rapidly that they could not be taken up by dog phagocytes mixed with them. For the anti-rat serum was not only hemolytic but an active stimulant to phagocytosis as well. And, had it been added to the phagocytic mixtures in a quantity which permitted the taking up of cells while hemolysis of them was going on, a proper comparison between the corpuscles in the phagocytes submitted to serum and those submitted to Locke's solution would have been impossible. The results of this test were so interesting that they will be given in detail.

---

[7] Levaditi, C., *Ann. de l'Inst. Pasteur*, 1902, xvi, 233.

TABLE III.

Mixture.	25 per cent rat red blood corpuscles.	Dog serum.	Exudate.	Anti-rat serum.	Apparent hemolysis.	Real condition as determined microscopically.
	cc.	*cc.*	*cc.*	*cc.*		
1	0.1	0.1	0.25	0.5	Complete within 5 min.	No phagocytosis. Complete hemolysis.
2	0.1	0.25	0.25	0.25	Complete. (?)	Considerable phagocytosis. All free red blood corpuscles hemolyzed.
3	0.1	0.25	0.25	0.25 of 50% solution.	+++	Profuse red sediment, of phagocytes enormously distended with red cells. All free red cells hemolyzed.
4	0.1	0.25	0.25	0.25 of 25% solution.	++	

Incubation was for 1 hour at 37°C.

Only in the first mixture was there complete hemolysis and in this all the red cells had suffered destruction within the first 5 minutes of incubation (Table III). In the other mixtures the degree of color of the supernatant fluid at the end of an hour indicated incomplete hemolysis as did the profuse red sediment. But these findings were not due to the serum's lack of hemolytic power, for, as the microscope showed, all the red cells remaining free had been hemolyzed. Many though, had been ingested by cells of the exudate, and thus were protected from hemolysis. The abundant red sediment consisted of phagocytes distended with red cells. Some of the phagocytes had extended only the thinnest layer of glassy cytoplasm over the red corpuscles which stood out, quite unhemolyzed, as knobs on their surface. Such corpuscles were evidently protected from the serum by their intracellular situation. But most of the ingested red cells had been much injured and had coalesced into orange-yellow globules (Fig. 1).

The test made it evident that in order to avoid phagocytosis in the presence of the hemolytic serum sufficient of this serum must be added to cause hemolysis of all the red cells within 5 minutes.

Now the main experiment was proceeded to. The following mixtures were made in a number of tubes.

Mixture 1.—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. 25 per cent dog serum.

Mixture 2.—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. 10 per cent dog serum.

At the end of an hour's incubation the mononuclear cells were found to have ingested numbers of apparently unchanged red cells,—from 6 to 25, as a rule.

To some of the duplicate tubes 0.5 cc. anti-rat dog serum was added, to others the same quantity of Locke's solution, and incubation was resumed. At the end of a further hour the preparations were examined for evidence of destruction of the intracellular red cells. None had occurred. The erythrocytes within the mononuclears submitted to anti-rat serum, like those within the phagocytes treated with Locke's solution, were still intact (Fig. 2). But the anti-rat serum had hemolyzed all extracellular erythrocytes and the phagocytes lay in the midst of masses of shadows.

A still more exacting test was carried out, as follows:

Mixture 3.—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. concentrated dog serum. After 1 hour's incubation 0.5 cc. anti-rat serum was added to some of the tubes, an equivalent amount of Locke's solution to others, and incubation continued for 2 hours more. In this instance the amount of serum used for opsonization caused injury to the red cells, of which some coalesced into globules immediately after their ingestion, though the majority remained intact. And now when the phagocytes full of these globules and of more or less injured and vulnerable cells were submitted to anti-rat serum and to Locke's solution, respectively, for 2 hours, no microscopic differences in their contents were observed. The anti-rat serum had been powerless to enhance the breaking-down of the red cells.

The dog red cells present in the exudate were far too few to constitute a source of error in the findings. Nevertheless, a number of control tests were made with a 25 per cent suspension of dog cells instead of rat cells. They were not phagocyted. In the experimental tests proper, the free dog cells were easily distinguished by their failure to agglutinate or hemolyze in the anti-rat serum.

The results of this experiment were clear-cut. The phagocytes protected red cells within them from the action of a powerful homologous antiserum (Figs. 2 and 3).

Protection a Function of the Living Cell.

The condition of the leukocytes exerting this protective action remained to be determined. Were they perhaps injured by the antiserum, despite the absence from it of agglutinins and hemolysins? The failure of the serum to penetrate could be explained in this way. Or was the protection a function of the living leukocytes and of living ones only? Tests with the material of Experiment 4 threw light on these points.

Experiment 5.—(*A*) Cells of the ultimate mixtures of Experiment 4 were examined with trypan-blue. The phagocytes which had been incubated with

anti-rat serum and those submitted to Locke's solution alike failed to take the stain. Many of the white cells that had failed to ingest red corpuscles showed nuclear staining.

(*B*) The following mixture was made up with the ingredients of Experiment 4:

0.1 cc. Locke's solution + 0.5 cc. washed exudate + 0.5 cc. concentrated anti-rat serum + 0.5 cc. dog serum.

After 1 hour's incubation the cells were separated out with the centrifuge and made up as follows:

0.5 cc. treated cells + 0.1 cc. 25 per cent suspension of red blood corpuscles + 0.5 cc. dog serum.

At the end of an hour profuse phagocytosis had taken place, proving that the leukocytes could not have been seriously injured by the antiserum.

(*C*) Portions of the ultimate mixtures of Experiment 4, in which phagocytosis had occurred, were kept in the ice box at about 2° C. and examined each day. At the end of the first 24 hours the cellular sediment had largely lost its ruddy color. The microscope showed that this was due to diffusion out of the phagocytes of pigment from the ingested red cells. The majority of the leukocytes had now a ground glass appearance. In Mixtures 1 and 2 there could be seen within the phagocytes the intact stromata of red cells from which the hemoglobin had disappeared. In Mixture 3 the stromata were not so clearly visible. In this instance, one will recall, the majority of the red cells were much damaged previous to ingestion. In all the mixtures there were still some phagocytes containing bright red cells, and in Mixture 3 some with orange-yellow globules. Phagocytes containing one or two intact cells and the shadows of others were not observed; but the protoplasm of many phagocytes was stained light orange, due to the seeping out of the hemoglobin from ingested red elements. With the trypan-blue test it was found that the cells containing bright erythrocytes or globules regularly failed to stain. So too did the cells tinted light orange. Practically all the other leukocytes underwent an immediate nuclear staining.

After 3 days in the ice box the results were identical except that living phagocytes were now rare.

This experiment proved that the protection exerted by the phagocytes in Experiment 4 was not due to injury, but on the contrary was associated with active cell life. When the phagocytes died they became permeable, allowing a rapid diffusion outwards of the hemoglobin from the ingested erythrocytes, as well as diffusion inwards of the trypan-blue stain. It seems highly probable from these facts, as well as from common knowledge of the differences in permeability between dead and living tissues, that when phagocytes die they must lose largely if not entirely their protective power.

DISCUSSION.

There are in the literature a number of detached observations which corroborate our findings. Bordet found that "cholera spirilla injected into the blood stream of cholera immune animals are taken up by the leukocytes even before they can be subjected to lysis by the circulating lytic antibodies."[8] And Metchnikoff, Levaditi,[7] Briscoe,[9] and others have shown that red cells injected into the previously immunized animal may be phagocyted before they can hemolyze. But so far as we are aware no attention has been paid to these indications of protection by cells. Such protection had no practical importance in the instances cited because the phagocytes themselves were capable of destroying the organisms they had ingested. In our experiments as well the phagocytes may have possessed this ability. The demonstration of protection by them is not thereby invalidated. For the ability of cells to protect ingested organisms from the action of outside agencies must be considered as entirely distinct from the disposition they ultimately make of these organisms.

It remains to be determined how far the protection of microorganisms by living tissue cells, especially cells incapable of killing the microorganisms, is important in disease processes. The phenomenon may have much to do with the survival in the animal body of organisms such as the leprosy bacillus which is so often found living within cells of the fixed tissues; and it may serve to explain in part the therapeutic difficulties in such instances. It may throw light, furthermore, on the formation of new disease foci at points of injury in individuals of high general resistance. For if an infective agent can be "walled off" from the action of the body fluids by the protoplasm of a single cell containing it, there is no reason why it should not be carried unharmed wherever this cell goes.

CONCLUSIONS.

1. Living phagocytes are able to protect ingested organisms from the action of destructive substances in the surrounding fluid, and even from a strong homologous antiserum.

[8] See Zinsser, H., Infection and Resistance, New York, 1914.

[9] Briscoe, J. C., *Jour. Path. and Bacteriol.*, 1908, xii, 66.

2. There is evidence that the protection by phagocytes is largely if not entirely conditioned on their being alive.

3. These facts should be taken into consideration in the study of diseases caused by infectious agents capable of living within tissue cells.

EXPLANATION OF PLATE 90.

Fig. 1. Red cells hemolyzing within phagocytes. There is a coalescence of the cells into globules. × 625.

Fig. 2. Intact red cells of the rat within dog phagocytes submitted for 1 hour to a powerful anti-rat dog serum. Many of the red cells appear pale because they are out of focus. The only free erythrocytes that have not been laked are a few dog cells. × 625.

Fig. 3. Red cells of the rat still intact within dog phagocytes submitted for 2 hours to a dog serum strong'y hemolytic for rat cells. The shadows of numerous erythrocytes hemolyzed while free are just visible. × 625.

THE EFFECT OF DIGITALIS ON THE NORMAL HUMAN ELECTROCARDIOGRAM, WITH ESPECIAL REFERENCE TO A-V CONDUCTION.

By PAUL DUDLEY WHITE, M.D., AND ROBERT RAY SATTLER, M.D.

(*From the Massachusetts General Hospital, Boston.*)

PLATES 91 TO 94.

(Received for publication, February 18, 1916.)

A study of the effect of digitalis on the normal human electrocardiogram was undertaken by us through a desire to throw more light on the significance of the various grades of heart block not infrequently produced in patients by only moderate amounts of digitalis. Little attention has hitherto been paid to the careful electrocardiographic study of the influence of digitalis on A-V conduction in the normal human heart.

Cohn and Fraser[1] in 1913 reported the study with the string galvanometer of twelve patients with normal cardiac rhythm, four of them without heart lesion. Digitalis in doses equivalent to 2 to 4 gm. of the leaves produced changes in A-V conduction in all the patients. A partial or complete return to the original conduction time was always produced by atropine. In 1914 Cohn[2] reported an investigation with digitalis of patients having an early stage of heart disease with normal mechanism. He concludes that "An effect on conduction may be set down as a usual effect of giving the drug, apart from specific preexisting injury." In our experiment with normal active young adults we have come to the same conclusion and have evidence to show that this defect in conduction is practically entirely due to increased tone and irritability of the vagus.

In this investigation five healthy young male adults were studied by us electrocardiographically. The Cambridge model of the Ein-

[1] Cohn, A. E., and Fraser, F. R., *Jour. Pharm. and Exper. Therap.*, 1913–14, v, 512.

[2] Cohn, A. E., *Jour. Am. Med. Assn.*, 1915, lxiv, 463.

TABLE I.

Table of Measurements of Case 1, Age 29 Years.

	Date.	Electrocardiogram.									Heart rate.	Blood pressure.		Subjective sensations.
		Intervals (sec.).			Amplitudes (10^{-3} volt).									
		P − R	R + S	R to end of T	P_2	R_2	S_2	T_2	T_1	T_3		Systolic.	Diastolic.	
	1915													
Normal (before digitalis)..........	Nov. 8	0.164	0.065	0.312	0.1	0.8	0.05	0.15	0.15	0	76	118	78	
After 0.3 gm. digitalis............	" 10				0.1	0.85	0.05	0.2	0.15	0	71			
" 0.6 " "	" 11				0.1	0.85	0.05	0.1	0.15	−Tr.	68	112	64	
" 0.9 " "	" 12				0.1	0.8	Tr.	0.1	0.15	−Tr.	68			
" 1.2 " "	" 13				0.1	0.85	"	0.1	0.1	0	64	124	72	Headache, anorexia.
" 1.5 " "	" 14	0.164			0.1	0.85	"	0.1	0.15	−0.05	69			
" 1.8 " "	" 15	0.187			0.1	0.8	0.05	0.1	0.15	−Tr.	64	122	72	
" 2.1 " "	" 16	0.186			0.1	0.85	Tr.	{−Tr. +0.05	0.05	{−Tr. +Tr.	64			Palpitation.
" 2.4 " "	" 17	0.186			0.05	0.8	0	{−0.05 +0.05	0.05	{−Tr. +Tr.	71	130	70	"
" 2.7 " "	" 18	0.203			0.05	0.85	0	{−Tr. +0.05	0.05	−0.05	70			" and anorexia.
" 3.0 " "	" 19	0.205	0.069	0.317	0.1	0.8	+Tr.	{−Tr. +Tr.	{−0.05 +Tr.	−0.05	77	124	70	Palpitation and nausea.
1 day after stopping digitalis....	" 20	0.222	0.072		0.1	0.9	∓ "	{−0.05 +0.05	0.1	∓Tr.	69			Arrhythmia, nausea.
2 days " " "	" 21	0.185			0.1	0.85	0	{−0.05 +Tr.	0.1	Tr.	80			Bigeminy at night.

3 days after stopping digitalis....	Nov. 22	0.201			0.1	0.9	Tr.	−0.05 +0.05	−0.05 +0.05	0	86		Acute cold (coryza).
4 " " " "	" 23	0.183			0.1	0.85	0	−Tr. +Tr.	−Tr. +0.05	0	88		Acute cold (coryza).
5 " " " "	" 24	0.217			0.1	0.85	0.2	−0.05 +0.1			77		
7 " " " "	" 26	0.186			0.1	0.85	Tr.	0.05	0.1	−0.05	69		
8 " " " "	" 27	0.172			0.05	0.75	0	0.05	0.1	−Tr.	75		
9 " " " "	" 28												Pauses felt in pulse until noon to-day.
10 " " " "	" 29	0.170			0.1	0.8	Tr.	0.15	0.1	Tr.	67		
12 " " " "	Dec. 1	0.172			0.1	0.85	0.05	0.1	0.1	0	69		
14 " " " "	" 3	0.164			0.1	0.9	Tr.	0.15	0.2	Tr.	64		
After 3.0 gm. digitalis.													
1 min. after exercise.........	Nov. 19	0.293	0.080		0.1	0.9	"	0.2	0.3	+Tr.	109		
45 " " atropine........	" 19	0.148	0.077	0.262	0.15	0.85	0	−0.05 +Tr.	−0.05 +0.05	− "	120		
Normal.	*1916*												
1 min. after exercise.........	Jan. 18	0.128			0.3±	0.8	0.2	0.3			160		
45 " " atropine........	" 10	0.138			0.1	0.85	0	0.15	0.15	−0.05	119		
	1913												
Normal control..................	Dec.	0.168			0.1	0.95	0.1	0.2	0.15	0.1	84		
	1915												
" "	Dec. 9	0.165			0.1	0.9	0.1	0.15	0.15	0	63		
	1916												
" "	Jan. 10	0.165			0.1	0.85	0.05	0.15	0.15	Tr.	80		
" "	" 18	0.158			0.1	0.8	∓ Tr.	0.15			75		

TABLE II.

Table of Measurements of Case 2, Age 27 Years.

	Date.	Electrocardiogram. Intervals (sec.). P – Q	Q+R +S	Q to end of T	Amplitudes (10^{-2} volt). P_2	Q_2	R_2	S_2	T_2	T_1	T_3	Heart rate.	Blood pressure. Systolic.	Diastolic.	Subjective sensations.
	1915														
Normal (before digitalis)........	Nov. 8	0.161	0.078	0.344	0.15	0.1	1.45	Tr.	0.3	0.15	0.15	67	130	70	
After 0.3 gm. digitalis..........	" 10				0.15	0.05	1.35	"	0.3	0.2	0.1	75			
" 0.6 " "	" 11				0.15	0.05	1.35	"	0.25	0.15	0.1	72	122	70	
" 0.9 " "	" 12				0.1	0.1	1.35	"	0.15	0.05	0.1	79			Palpitation.
" 1.2 " "	" 13				0.15	0.1	1.45	"	0.1	Tr.	−0.05 / +0.05	87	116	82	Malaise.
" 1.5 " "	" 14	0.161			0.1	0.1	1.4	"	0.1	0.05	0.05	75			
" 1.8 " "	" 15	0.173			0.1	0.05	1.3	"	0.05	0.1	−0.05	80	102	70	Headache, malaise.
" 2.1 " "	" 16	0.166			0.15	0.1	1.4	"	0.1	0.1	−Tr. / +Tr.	73			Malaise.
" 2.4 " "	" 17	0.170			0.1	0.05	1.35	"	−Tr. / +0.1	0.1	−Tr. / +Tr.	64	112	70	Headache.
" 2.5 " "	" 18	0.172	0.078	0.310?	0.1	0.05	1.35	"	−Tr. / +0.1	0.05	−0.05 / +Tr.	66	106	70	"
1 day after stopping digitalis..	" 19	0.167			0.15	0.05	1.2	0.05	0.1	0.05	−Tr. / +0.05	84			
3 days " " " ..	" 21	0.167			0.1	0.05	1.35	Tr.	0.15	0.1	−Tr. / +0.05	75			
5 " " " " ..	" 23	0.172			0.15	0.05	1.4	0	0.2	0.15	0.05	62			
8 " " " " ..	" 26	0.162			0.1	0.05	1.4	Tr.	0.2	0.1	0.05	75			

12 days after stopping digitalis..	Nov. 30				0.15	0.05	1.35	Tr.	0.2	0.05	0.1	70		
19 " " " " ..	Dec. 7				0.15	Tr.	1.5	0.2	0.3	0.15	0.15	70		
After 2.5 gm. digitalis.														
1 min. after exercise......	Nov. 18	0.183	0.082	?	0.2	0.1	1.3–1.6	0.15	0.35	0.2	0.15	118		
45 " " atropine.....	" 18	0.144	0.081	0.259	0.15	0.1	1.4	Tr.	−0.1 +0.05	0.1	−0.1	120		
Normal.	*1916*													
1 min. after exercise.......	Jan. 10	0.152			0.2	0.2	1.6+	0.1	0.55			136		
45 " " atropine......	" 10	0.137			0.15	0.1	1.5	0.1	0.2	0.15	Tr.	128		
Normal control................	" 10	0.157								0.1	0.1			
" "	" 18	0.155										83		
	1915													
" "	May 10	0.170												

thoven string galvanometer was used with non-polarizable electrodes. Photographic plates containing the three leads of Einthoven were taken before the administration of the drug, at approximately 24 hour intervals during the administration and at intervals of a day or two after the drug was stopped until the electrocardiograms had returned to normal. We have used Caesar and Loretz digitalis leaf in amounts ranging from 2 to 3 gm. at the rate of 0.3 gm. daily. Different amounts of digitalis were used in order to compare the durations of the drug effects. The effects of atropine (0.002 gm. subcutaneously) and exercise (a fast run of about one-quarter of a mile) on the normal and on the digitalized electrocardiograms were studied. Several control records were taken in order to determine the normal range of A-V conduction time in the individuals tested.

Measurements were made by projecting the images on the photographic plates upon a glass screen at a magnification of twenty-five diameters. For this purpose a microphotographic apparatus was used. The electrocardiographic intervals were measured off by calipers on a scale of one-sixtieth of an inch and compared with the measurements of the time intervals. Our maximum error is below 0.01 second. Time intervals of 0.2 second were used instead of smaller intervals, such as 0.04 second, because of the greater accuracy of measurement. In work which one of us did with Lewis[3] on the measurement of P-R intervals in experimental curves it was found that the upstrokes of deflections so often fell upon and were obscured by the time lines separating intervals of 0.04 second that these small intervals were given up and 0.2 second time intervals adopted. Three beats were measured on each plate and their average was recorded in the final tables (Tables I to V).

In addition to the determination of the A-V conduction time as obtained from the P-R or P-Q interval (the latter if a Q is present), the Q-end of S and Q-end of T intervals before and after digitalis have been measured; the digitalis effects on the amplitudes of the electrocardiographic deflections, on the heart rates, on the blood pressures, and on the subjective sensations have been studied. On account of the fact that the tension of the string was not always

[3] Lewis, T., and White, P. D., *Heart*, 1914, v, 335.

accurately adjusted allowance for errors has been made in calculating the curves.

Tables I to V contain the measurements of the A-V conduction times as expressed by the P-Q or P-R intervals, the measurements of the Q-end of S and the Q-end of T intervals, the amplitudes of the deflections, the heart rates, the blood pressures, and the subjective sensations. Table VI contains the effects of exercise on A-V conduction in the normal electrocardiogram. Figs. 1 and 2 show the control electrocardiograms of the five subjects and those taken at the end of the digitalis administration. Fig. 3 shows the atropine and exercise effects on the digitalized electrocardiograms. Figs. 4 and 5 illustrate the phenomenon resulting from digitalis in one of the subjects.

DISCUSSION.

A-V Conduction.

Digitalis caused a delay in A-V conduction in four of our five subjects. In three the lengthening was but slight and hardly greater than the normal range of conduction time in these same individuals (Tables II, III, and IV). In none of these three did the P-R interval equal or exceed 0.2 second. In the first subject (Table I) prolongation of conduction time up to 0.3 second occurred after 3.0 gm. of digitalis, but in no one of the five did the P-R interval increase to as much as 0.2 second after the ingestion of 2.5 gm. or less of digitalis. In every instance after digitalis even when the delay in conduction amounted to more than 0.05 second atropine reduced the P-R interval to less than its original value. The increased vagal action occurring with the rapidly slowing pulse after exercise added to the defect already present after digitalis. Normally we have found that immediately after exercise the A-V conduction time is markedly decreased, more even than it is decreased normally by atropine, 0.002 gm. subcutaneously (Tables I, II, and VI). Our maximum shortening was from 0.158 to 0.112 second. Shortening of the P-R interval after exercise was found by Lewis and Cotton[4] in 1913.

[4] Lewis, T., and Cotton, T. J., *Jour. Physiol.*, 1913, xlvi, p. lx.

TABLE III.

Table of Measurements of Case 3, Age 28 Years.

	Date.	Electrocardiogram.										Heart rate.	Blood pressure.		Subjective sensations.
		Intervals (sec.).			Amplitudes (10^{-3} volt).										
		P−Q	Q+R+S	Q to end of T	P_2	Q_2	R_2	S_2	T_2	T_1	T_3		Systolic.	Diastolic	
	1915														
Normal (before digitalis).........	Nov. 8	0.135	0.104	0.379	0.05	0	1.1–1.25	0.3	0.2	0.25	−0.05 +Tr.	68	122	70*	
After 0.3 gm. digitalis...........	" 10				0.1	Tr.	1.25	0.35	0.2	0.25	−0.05 +Tr.	60			
" 0.6 " "	" 11				0.15	0.05	1.45	0.35	0.1	0.25	−0.1 +0.05	73	125	68	
" 0.9 " "	" 12				0.1	Tr.+	1.3	0.35	−Tr. +0.05	0.15	−0.1 +0.05	73			
" 1.2 " "	" 13	0.134			0.05	0.05	1.3±	0.35±	0.1	0.15	−0.1 +Tr.	70	134	78	
" 1.5 " "	" 14	0.149			0.1	0.05	1.4±	0.35±	−0.05 +0.1	0.15	−0.1 +Tr.	63			
" 1.8 " "	" 15	0.154			0.1	0.05	1.3±	0.3	−0.05+ +0.05	0.15	−0.1 +Tr.	64	140	62	
" 2.1 " "	" 16	0.152			0.1	Tr.	1.35±	0.3	−0.05 +0.1	0.15	−0.1 +Tr.	70			
" 2.3 " "	" 17	0.156	0.108	0.367	0.05	"	1.3±	0.3	−0.05 +0.05	0.15	−0.15	57	126	64	Slight nausea.
1 day after stopping digitalis....	" 18	0.150			0.1	0.05	1.35±	0.35	−0.1 +0.05	0.15	−0.2 +0.1	63			

3 days after stopping digitalis....	Nov. 20	0.149			0.1	Tr.	1.4±	0.35	−0.05 +0.1	0.2	−0.2	75		
5 " " " "	" 22	0.154			0.1	0	1.3±	0.35±	−0.05 +0.1	0.15	−0.15	67		
7 " " " "	" 24	0.148			0.1	0	1.25−	0.35±	−Tr. +0.1	0.2	−0.15 +Tr.	66		
9 " " " "	" 26	0.159			0.1±	Tr.	1.2	0.35±	−Tr. +0.1	0.2	−0.15	74		
12 " " " "	" 29	0.135			0.05	0	1.25	0.35±	0.2−	0.25	−0.05 +Tr.	59		
14 " " " "	Dec. 1				0.05	0	1.2	0.3	0.15	0.2	−0.1	64		
After 2.3 gm. digitalis.														
1 min. after exercise.........	Nov. 17	0.151	0.100	0.290	0.25	0.05	1.0–1.3	0.3–0.45	0.3	0.15	−0.05 +0.1	113		
45 " " atropine.........	" 17	0.144	0.087	0.314?	0.2	0.1	1.3+	0.3	−0.1 +0.05	0.1	−0.2	116		
Normal control.................	Dec. 6	0.144			0.1	0	1.4	0.4−	0.15+	0.25	−0.05+ +Tr.	65		
" "	" 8				0.1	Tr.	1.3±	0.3–0.35	0.2	0.3	−0.05 +Tr.	55		
" "	*1916* Jan. 10	0.141			0.1	0	1.3±	0.35±	0.2	0.25	−0.05 +Tr.	75		

* Blood pressure estimation made Nov. 9, 1915, after 0.1 gm. digitalis.

The U deflection in this subject is unusually pronounced but is unaffected by digitalis. As the T wave flattens it becomes at length level with the U wave.

From the effect of digitalis on A-V conduction in normal hearts it seems to us reasonable to conclude that there is either an abnormal irritability of the vagus or a damage to the conduction tissue itself, if heart block, even a delay in conduction beyond a P-R interval of 0.2 second, occurs in patients after small or moderate amounts of an active preparation of digitalis (for example 1.0 to 2.0 gm. of Caesar and Loretz standardized leaves in 4 to 7 days). If, however, this drug is continued up to and beyond 3.0 gm., a slight defect in A-V conduction then appearing for the first time may be reasonably ascribed to the digitalis and no blame be placed on the conducting tissue.

Arrhythmia.

The greater action, or at least the less balanced action, of the vagus at night slowed the heart rate in two of the subjects (Cases 3 and 1) below the usual rate, in one the pulse falling as low as 48 to the minute; in the other subject the vagal activity was still further evidenced by the occurrence of blocked auricular premature beats—a phenomenon dependent on delay in conduction time (Figs. 4 and 5). This arrhythmia, the only one produced in any of the subjects, began to appear after 3.0 gm. of digitalis had been taken. As far as we are aware it is the first recorded observation of such a result from digitalis. It consisted of an interruption of the normal rhythm by premature ectopic auricular contractions (almost isoelectric in the electrocardiogram) without ventricular response. Polygrams and electrocardiograms of the phenomenon were obtained with considerable difficulty because of the fact that the irregularity almost always occurred late at night, apparently when the influence of the vagus was greatest and tended to disappear if an attempt was made to obtain graphic records. At times it occurred so often as to produce a bigeminy—two normal beats followed by a premature auricular contraction during and after which there was a pause in the pulse. This irregularity first appeared when the influence of digitalis was at its maximum, as shown by the P-R interval (0.222 second) 1 day after stopping digitalis. It occurred off and on for the following 8 days and nights, but since the 9th day after stopping digitalis (3 months ago) it has not once occurred. The subject had never had premature beats so far as known prior to the ingestion of the

TABLE IV.

Table of Measurements of Case 4, Age 24 Years.

	Date.	Electrocardiogram.										Heart rate.	Blood pressure.		Subjective sensations.
		Intervals (sec.).			Amplitudes (10^{-3} volt).										
		P–Q	Q+R+S	Q to end of T	P_2	Q_2	R_2	S_2	T_2	T_1	T_3		Systolic.	Diastolic.	
	1915														
Normal (before digitalis)	Nov. 9	0.132	0.080	0.303	0.15	Tr.	0.95–1.0	0.35	0.3	0.2	0.1	85	140	100	
After 0.6 gm. digitalis	" 11				0.15	"	1.0	0.3–0.35	0.25	0.15–0.2	0.05±	80	144	100	
" 0.9 " "	" 12				0.15	"	1.0–1.05	0.35±	0.2	0.15	0.05	83			Malaise.
" 1.2 " "	" 13				0.1	"	0.95	0.35	0.2	0.15	0.05	80	122	86	" and headache.
" 1.5 " "	" 14	0.133			0.15	"	1.05–1.1	0.25–0.3	0.2–	0.1+	0.05+	82			Palpitation.
" 1.8 " "	" 15	0.140			0.15	"	0.9–0.95	0.3–0.35	0.2–	0.15	0.05–	83	138	90	Dizziness and anorexia.
" 2.0 " "	" 16	0.150	0.075	0.301	0.15	"	1.0–1.05	0.35±	0.25	0.15	0.1	83	128	90	
1 day after stopping digitalis	" 17	0.147			0.1	0	0.95–1.0	0.3	0.2+	0.2	0.05	81			
3 days " " "	" 19	0.140			0.15	Tr.	0.95	0.3	0.2	0.15	0.05	76			
5 " " " "	" 21	0.142			0.15	"	1.1	0.35	0.25	0.2	0.1	88			
7 " " " "	" 23	0.134			0.15	"	1.05	0.35	0.2	0.1+	0.1–	86			
11 " " " "	" 27				0.15	"	1.05±	0.3	0.25	0.2	0.05	86			
14 " " " "	" 30				0.15	0	1.1±	0.3±	0.3	0.2	0.1±	75			
After 2.0 gm. digitalis.															
{ 1 min. after exercise	" 16	0.155	0.090	0.273?	0.25±	0.05	1.0	0.65–0.8	0.7	0.2	0.4	140			
{ 20 " " atropine	" 16	0.124	0.082	0.263?	0.25	0	0.9–0.95	0.5	0.3	0.15	0.15	138			
Normal control	Dec. 3				0.1	Tr.	0.95–1.0	0.3	0.3	0.25	0.1	79			

TABLE V.

Table of Measurements of Case 5, Age 32 Years.

	Date.	Electrocardiogram. Intervals (sec.).			Electrocardiogram. Amplitudes (10^{-3} volts).							Heart rate.	Blood pressure.		Subjective sensations.
		P − Q	Q + R + S	Q to end of T	P_2	Q_2	R_2	S_2	T_2	T_1	T_3		Systolic.	Diastolic.	
	1915														
Normal (before digitalis).......	Nov. 8	0.138	0.081	0.322	0.2	0.1	1.65±	0.2	0.3+	0.1	0.25+	76	116	82	
After 0.3 gm. digitalis.........	" 9				0.15	0.1	2.0+?	0.2	0.35+?	0.2	0.2+	62			
" 0.6 " "	" 10				0.15	0.1	1.65−	0.15–0.2	0.25	0.15	0.1−	78			
" 0.9 " "	" 11				0.2	0.1	1.65±	0.2−	0.25+	0.2	−Tr. +0.1	72	122	70	
" 1.2 " "	" 12				0.15	0.1	1.7±	0.15	0.2	0.15	−Tr. +0.1	78			
" 1.5 " "	" 13				0.15	0.1	1.6±	0.15–0.2	0.15	0.05	−0.1 +0.1	77	125	70	
" 1.8 " "	" 14				0.15	0.05	1.6−	0.15–0.2	0.15±	0.1	−0.1 +0.05±	67			Slight anorexia, nausea, dizziness.
" 2.0 " "	" 15	0.137	0.080	0.310	0.15	0.1	1.6−	0.15–0.2	−Tr. +0.2	0.1	−0.05 +0.1	78	122	70	Slight anorexia, nausea, dizziness.

1 day after stopping digitalis...	Nov. 16	0.136			0.2	0.1	1.6–1.75	0.25±	−0.05 +0.15	0.05	−0.1 +0.1	88		
3 days " " " ...	" 18				0.15	0.1–0.15	1.7±	0.2±	−Tr. +0.2	0.15	−0.05 +0.1	78		
5 " " " " ...	" 20				0.15	0.1	1.6	0.2	0.25	0.15	−Tr. +0.15	74		
7 " " " " ...	" 22				0.15	0.1	1.7±	0.2	0.2	0.15	−0.05 +0.1	76		
9 " " " " ...	" 24				0.15	0.1	1.45–1.75	0.2	0.15–0.2	0.1	−Tr. +0.05	83		
11 " " " " ...	" 26				0.15	Tr.	1.65	0.2	0.25	0.25	0.05	67		
14 " " " " ...	" 29				0.15	Tr.	1.55–1.8	0.2	0.3+	0.25−	0.1−	76		
After 2.0 gm. digitalis.														
45 min. after atropine..........	" 15	0.123	0.081	?	0.2	0.2±	1.3±	0.2–0.25	−0.1+ +0.1−	0.05	−0.2 +0.05	124		
Normal control................	Dec. 6	0.140										75		
" "	*1916* Jan. 9	0.141										75		

drug. For these reasons and because of the fact that it was directly dependent on a certain degree of prolongation of the P-R interval (to 0.295–0.300 second) the arrhythmia can be ascribed to digitalis. The subjective sensations of the irregularity were interesting, for the pause following the ectopic auricular beat could always be foretold by the feeling of the premature auricular systole itself consisting of a wave of fulness rising in the neck and throat. The association of mechanical activity of the auricle with the abnormal deflection in the electrocardiogram is clearly shown in the jugular tracing (Fig. 5 b) taken by Dr. O. F. Rogers, Jr. The production of the premature auricular systole by a mechanical stimulus from the contracting ventricle would at present best explain the fact that the R-P interval is much shorter than the P-R interval just preceding and that it varies little if at all in length.

Amplitudes of Electrocardiographic Deflections.

T Wave.—Cohn and Fraser[1] reported in 1913 their observations that the T wave of the human electrocardiogram was inverted in many of their patients who were under the influence of digitalis. More recently Cohn, Fraser, and Jamieson[5] have shown that digitalis given by mouth to patients began to cause a change in shape and amplitude of the T wave as early as 36 to 48 hours after the administration of the drug had begun, the change increasing as the digitalis was continued and persisting for from 5 to 22 days after the drug had been stopped. It is interesting to note that in all five of our entirely normal subjects, as a result of digitalis the T wave was decreased in amplitude in every lead. There seemed to be no direct connection between the effects of digitalis on the conduction time and on the T wave as indicated particularly well by one subject (Table V), who suffered no defect in A-V conduction but who did show a considerable decrease in the amplitude of T (Fig. 1). We have found that the first electrocardiographic evidence of digitalis and, for that matter, the first evidence, of any sort, of digitalis action is the decrease in the amplitude of the T deflection in the case of normal individuals.

[5] Cohn, A. E., Fraser, F. R., and Jamieson, R. A., *Jour. Exper. Med.*, 1915, xxi, 593.

TABLE VI.

The Effect of Exercise on Normal Electrocardiograms of Cases 1 and 2.

		P-Q interval.	Heart rate.
Case 1.	Normal (before exercise)	0.158	75
	½ min. after exercise	0.112	178
	1 " " "	0.128	160
	2 " " "	0.167	111
	8 " " "	0.176	95
Case 2.	Normal (before exercise)	0.150±	90
	¼–½ min. after exercise	0.117	155
	¾ " " "	0.132	145
	1 " " "	0.144	135
	2 " " "	0.150	115
	5 " " "	0.153	99
	10 " " "	0.149	102
	30 " " "	0.156	86

Effect of Exercise and Atropine on the Digitalized T Deflection.

The mechanism by which exercise acts on the heart temporarily removed the traces of the digitalis action on the T wave (Fig. 3), while atropine, 0.002 gm. subcutaneously, actually increased the digitalis effect on this deflection (Fig. 3) although the pulse rate was raised about equally by both procedures. Just the opposite action of these two tests was noted on the digitalized P-R interval.

P_2, Q_2, R_2, and S_2 Deflections.—These showed no clear-cut changes in amplitude as the result of the digitalis in our subjects.

SUMMARY.

Digitalis was given by mouth to five normal young male adults in amounts ranging from 2.0 to 3.0 gm. of standardized leaves in the course of 7 to 10 days. The As-Vs interval was prolonged in four of the five subjects, the greatest prolongation occurring in the case of the subject who received the most digitalis and none at all in one who received only 2.0 gm. There was no prolongation to so great an interval as 0.2 second until 2.7 gm. had been taken. The effects of the digitalis on conduction time began 5 to 6 days after the drug had been started and after 1.5 to 1.8 gm. had been taken. The

effects persisted for 1 to 2 weeks after the drug had been stopped. Atropine removed completely the effect of digitalis on A-V conduction. The slowing heart rate after exercise was accompanied by an enhancement of the defect in conduction. The change in conduction through digitalis was therefore almost entirely, if not entirely, due to increase of vagal tone and irritability.

Digitalis did not affect to an appreciable extent the Q-end of S and the Q-end of T intervals. Exercise and atropine both shortened the ventricular complex Q-end of T while the subject was under digitalis.

The amplitude of the T wave, especially in Lead II, was changed within 48 hours after digitalis had been started, a decrease then beginning which became greater as the drug was continued and which persisted until 10 to 19 days after the digitalis had been stopped. The change in the T deflection preceded by several days the change in conduction time. The T wave, therefore, in the normal subject as well as in the patient gives us the earliest indication of digitalis action.

The amplitudes of P, Q, R, and S were not materially influenced by the amounts of digitalis given.

The pulse rate in two subjects became lower than usual at night as the result of the digitalis; otherwise there was no evidence of vagal action on the sino-auricular node. Blood pressure was uninfluenced by the digitalis. Mild subjective sensations occurred in all the subjects during the administration of the drug.

A curious, hitherto undescribed, digitalis arrhythmia consisting of blocked auricular premature beats occurred in one subject after 3.0 gm. of digitalis had been taken.

Supplementary Note.—A second series of five healthy young male adults has recently (March, 1916) been studied by one of us electrocardiographically during a course of digitalis —2.6 to 3.3 gm. Caesar and Loretz leaf in 9 to 11 days. (The drug was weaker than that used in the previous investigation.) Three subjects showed slight prolongation of the P-R interval but no bradycardia; the other two subjects, who had no delay in A-V conduction, showed a marked total bradycardia after the digitalis (heart rate of 43 in each in the middle of the forenoon). The T_2 deflection of the electrocardiogram

was more or less flattened by the digitalis in all five subjects. Atropine given subcutaneously at the height of the digitalis action depressed the T wave still further in every instance.

EXPLANATION OF PLATES.

In all figures abscissæ equal 0.2 second and ordinates equal 10^{-4} volts.

Where the galvanometer string has been too slack or too tense, the error is seen in the control deflection. The proper correction in the amplitude has been made. Amplitudes have been estimated to 0.05 of a millivolt.

Plate 91.

Fig. 1. Electrocardiograms (Lead II) of Case 5 (*A*), Case 4 (*B*), Case 3 (*C*), and Case 1 (*D*). The left hand column contains control records; right hand column contains records taken during full effect of digitalis. In the center below is a record of Lead II of Case 1 taken 1 day after digitalis had been stopped and showing the variation in the length of the P-R interval.

Plate 92.

Fig. 2. The three electrocardiographic leads of Case 2 before and immediately after the course of 2.5 gm. of digitalis. The change in the T deflection in each lead is evident.

Plate 93.

Fig. 3. Electrocardiograms (Lead II) of Case 4 (*A*), Case 3 (*B*), Case 2 (*C*) and Case 1 (*D*) after atropine (left hand column) and after exercise (right hand column) at the completion of digitalis. In the center below is the record of Lead II of Case 5 after atropine at the completion of 2.0 gm. of digitalis.

Plate 94.

Fig. 4. Lead II of Case 1 showing blocked auricular premature beats following gradual prolongation of the P-R interval. Electrocardiogram taken 5 days after the completion of 3.0 gm. of digitalis.

Fig. 5. Radial pulse tracing (*a*) and polygram (*b*) of Case 1 taken by Dr. O. F. Rogers, Jr., showing arrhythmia produced by the auricular premature beats which occurred as the result of 3.0 gm. of digitalis. *a'*, evidence in jugular pulse of blocked auricular premature beat. Time interval, 0.2 second.

THE INFLUENCE OF TETHELIN, AND OF OTHER ALCOHOL-SOLUBLE EXTRACTIVES FROM THE ANTERIOR LOBE OF THE PITUITARY BODY, UPON THE GROWTH OF CARCINOMATA IN RATS.

By T. BRAILSFORD ROBERTSON, Ph.D., and THEODORE C. BURNETT, M.D.

(*From the Rudolph Spreckels Physiological Laboratory of the University of California, Berkeley.*)

(Received for publication, February 29, 1916.)

Preparation of the Substances Employed.

One of us has recently succeeded in isolating the growth-controlling principle (tethelin, from τεθηλώς, growing) from the anterior lobe of the pituitary body. The methods of isolating the substance, its chemical properties and physiological actions, and the evidences of its identity with the growth-controlling principle are contained in articles which have been published elsewhere.[1] Briefly summarized, however, the method of isolating the substance is as follows: The anterior lobes of ox pituitaries are carefully separated from the posterior lobes and connecting parts and stripped of their connective tissue capsules. The tissue is then ground up in a mortar with three times its weight of a mixture of equal parts by weight of anhydrous sodium and calcium sulphates. The mixture is then dried on a water bath until nearly white, returned to the mortar, and thoroughly pulverized.

The pulverized material is then extracted with boiling absolute alcohol in an extraction apparatus (of the Bailey-Walker type) so constructed as to carry out the extraction at or near the temperature of boiling alcohol. The extraction is continued for 48 hours. The solution thus obtained is evaporated under reduced pressure until solid material begins to separate out on cooling. To this con-

[1] Robertson, T. B., *Jour. Biol. Chem.*, 1916, xxiv, 397, 409.

centrated solution is added one and one-half times its volume of dry ether. The substance is thus precipitated and, after washing in large volumes of alcohol-ether mixture containing alcohol and ether in the above mentioned proportions, may be dried over sulphuric acid at low temperatures and pulverized in a dry atmosphere.

The properties of tethelin may be summarized as follows: It is markedly hygroscopic, absorbing water rapidly from moist air. On standing after pulverization in contact with air in the presence of traces of moisture it darkens perceptibly in color and its iodine absorption value decreases. This decomposition is accelerated by warming. If packed in evacuated glass tubes, however, and perfectly dry, it may be heated to 80°C. without any perceptible discoloration.

Tethelin is soluble in water to the extent of about 5 per cent. It is also soluble in alcohol, ether, chloroform, and carbon tetrachloride. It is insoluble in a mixture of one part by volume of absolute alcohol and one and one-half parts by volume of dry ether. It contains 1.4 per cent of phosphorus, and nitrogen in the proportion of four atoms of nitrogen for every atom of phosphorus, two of the atoms of nitrogen being present in amino groups and one in an NH group which is converted into an amino group by hydrolysis with barium hydroxide. It yields unsaturated fatty acid soaps of barium on hydrolysis with barium hydroxide, and among the products yielded by hydrolysis with barium hydroxide followed by hydrolysis with dilute sulphuric acid is found *i*-inosite (hexahydroxybenzene).

Tethelin probably contains an imineazolyl group, and to this extent may be regarded as being related to the physiologically active substances of the posterior lobe of the pituitary body.[2] It is not, however, possessed of the characteristic physiological activity of these substances, relatively large doses administered intravenously to rabbits (50 mg. per kilo of body weight) producing only a very slight transient fall in blood pressure and no diuresis.

The action of tethelin upon the normal growth of mice is identical with the action of the whole anterior lobe.[3] It consists in a marked

[2] Barger, G., and Dale, H. H., *Jour. Physiol.*, 1910–11, xli, 499. Dale, H. H., and Laidlaw, P. P., *Jour. Physiol.*, 1911, xliii, 182. Aldrich, T. B., *Jour. Am. Chem. Soc.*, 1915, xxxvii, 203.

[3] Robertson, T. B., *Jour. Biol. Chem.*, 1916, xxiv, 385, 397.

retardation of the early (preadolescent) growth in weight (subsequent to 4 weeks after birth) and an equally marked acceleration of post-adolescent growth.

In a previous article[4] we have shown that the hypodermic administration of emulsified tissue of the anterior lobe of the pituitary body to rats, either directly into or in localities remote from the tumors, leads to a remarkable acceleration of the growth of the Flexner-Jobling carcinoma, especially during the period of growth between the 20th and 37th days succeeding inoculation. This effect is specific, since similar administrations of liver tissue, during the same period, far from causing any acceleration of the growth of the tumors, actually resulted in a slight but definite retardation of their growth.

In view of these results it appeared of importance to ascertain whether tethelin also reproduces the effect of the whole anterior lobe upon the growth of carcinomata, and to that end the investigations which are about to be described were undertaken. At the same time it seemed advisable to ascertain whether any other alcohol-soluble extractive of the anterior lobe of the pituitary body exerts any action upon the growth of carcinomata. Three such fractions were prepared and their action upon the growth of carcinomata was investigated.

Fraction A.—Tethelin was removed from the alcohol extract by precipitation by ether as described above, and the supernatant fluid was syphoned off from the precipitate and filtered. The ether with part of the alcohol was then distilled off at atmospheric pressure (without exposure to the air) until the boiling point of the mixture reached 78° C. The solution, much diminished in volume, was now cooled by standing in the ice chest for a couple of days. A small quantity of material, slightly soluble in cold alcohol and only partially soluble in ether, was deposited. This was washed in cold alcohol and dried over sulphuric acid. This substance, which behaved like lecithin in forming emulsions with water, composed Fraction A. It probably consisted in great part of a mixture of phospholipins.

Fraction B.—The solution obtained after the separation of Fraction A was evaporated to dryness on a water bath in a vessel provided only with a small exit for the alcohol vapor. The residue was taken up in a small quantity of ether, in which it was completely soluble, and several volumes of acetone were added to this solution. The precipitate was collected on a filter, washed with

[4] Robertson, T. B., and Burnett, T. C., *Jour. Exper. Med.*, 1915, xxi, 280.

acetone and dried over sulphuric acid. This formed Fraction B; it consisted, probably entirely, of lecithins.

Fraction C.—The filtrate from the above was evaporated to dryness in a water bath in a vessel provided only with a small exit for the ether-acetone vapor. The residue, a dark colored substance, gummy when cool and soft and viscous when warmed, was dried over sulphuric acid. This formed Fraction C. It probably consisted largely of ordinary fats and also must have contained all the cholesterol and cholesterol esters originally present in the tissue. On triturating with water it formed an unstable emulsion.

From a number of successive batches of 300 anterior lobes, each weighing approximately 450 gm. (fresh tissue), were obtained the following approximate yields of the above described substances.

	gm.
Tethelin	2.6–3.0
Fraction A	0.3–0.8
Fraction B	4.0–6.0
Fraction C	4.0–6.0

History of the Tumors Employed.

We propagated the Flexner-Jobling carcinoma by inoculation into the axillary region through five generations. The percentage of takes was high, varying between 60 and 90 per cent. Half grown or adult animals were employed to propagate the tumors and also in the experiments enumerated below, with the exception of a small number of younger animals evenly distributed among all the different experimental groups. As stated in our previous communication referred to above, this tumor, non-metastasizing when supplied to us by Dr. Peyton Rous, yielded metastases in the first generation in this laboratory and has continued to do so in succeeding generations.

Influence of Tethelin upon the Growth of Carcinomata.

73 white rats were inoculated in the axillary region with peripheral portions of a large rapidly growing tumor of our 5th generation from Rous's 21st generation. After 21 days 49 of the animals (67 per cent) were found to have well developed tumors. These were divided into two batches. One, consisting of 24 animals was retained as controls, and the 25 individuals comprising the other received, on the 21st, 23rd, 25th, 28th, 30th, and 32nd days after inoculation, 0.6 cc. each of a 5 per cent solution of tethelin in $\frac{M}{6}$ sodium chloride, to which had been added 0.4 per cent of tricresol. The tethelin was injected hypodermi-

cally on the side remote from the tumor. The dose employed (30 mg.) corresponded to three anterior lobes, estimating the tethelin content of each anterior lobe to be 10 mg. No ill effects were observed to follow the administrations.

The tumors were measured through the skin in two diameters at right angles to each other on the dates enumerated below (Table I).

The mean of the two diameters (usually the longest and shortest) was recorded as the average diameter of any given tumor, and the average of these estimates was regarded as the average diameter of the tumors in any given group of animals.

The average diameter of the tumors in the control animals on the 21st day after inoculation was 12.4 mm., that of the tumors in the animals reserved for treatment was 12.1 mm. Calling these initial diameters in each group 100, and referring each of the subsequent measurements to this unit of comparison, we obtained the following results.

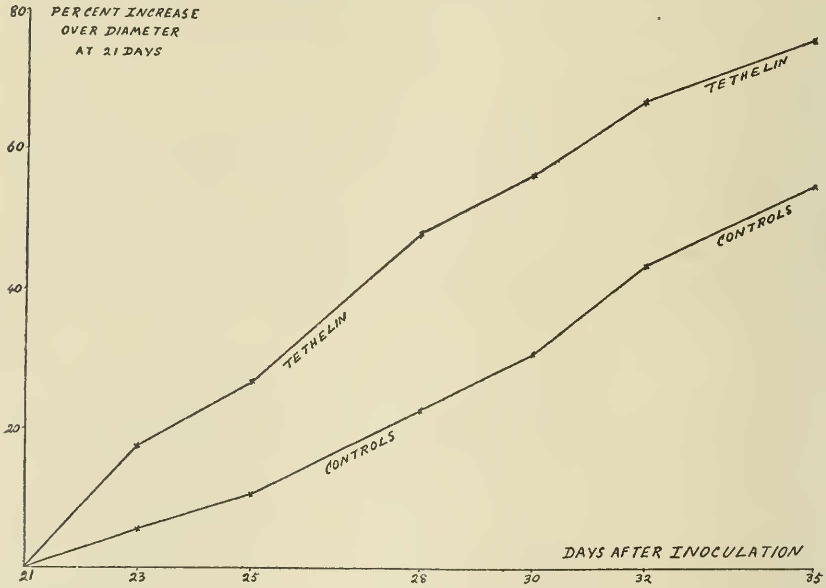
TABLE I.

Days after inoculation.	Diameter of tumors in controls.	Diameter of tumors in animals receiving tethelin.
21	100.0	100 0
23	105.6	117.4
25	110.5	126.4
28	122.6	147.9
30	130.6	156.2
32	143.5	167.0
35	154.9	176.0

These results are depicted graphically in Text-fig. 1. It will be seen that the administration of tethelin caused marked acceleration of the growth of the primary tumors, their linear growth between the first and fifth administration being from two to three times as rapid as that of the controls.

On the 63rd day all the surviving animals (23 of each group) were killed and the viscera examined for metastases. 2, or 9 per cent, of the control animals were found to have developed metastases, while 8, or 35 per cent, of the treated animals had developed metastases. The administration of tethelin, therefore, markedly enhances

the tendency of the tumor to form metastases, the differing result obtained by us with whole anterior lobe administration[4] being probably attributable to the relatively much smaller doses (only 0.5 gm., one-third of an anterior lobe in each dose) employed by us in our previous experiments.



TEXT-FIG. 1. The acceleration of the growth of carcinomata by hypodermic administrations of tethelin.

The Influence of Other Alcohol-Soluble Extractives of the Anterior Lobe of the Pituitary Body upon the Growth of Carcinomata.

140 white rats were inoculated in the axillary region with peripheral portions of a large tumor taken from one of the controls in the preceding experiments; *i.e.*, a tumor of our 6th generation from Rous's 21st generation. After 21 days 105 of the animals (75 per cent) were found to have well developed tumors. These were divided without selection into four batches. One, consisting of 27 animals, was retained as controls; the remaining three groups, consisting of 26 animals each, were treated with Fractions A, B, and C, respectively, on the 21st, 23rd, 25th, 28th, 30th, and 32nd days after inoculation. In each case the substance was administered hypodermically on the side of the animal remote from the tumor. Watery emulsions in $\frac{M}{6}$ sodium chloride solution containing 0.4 per cent of tri-

cresol were employed. The emulsion of Fraction A contained 1.7 per cent of the substance, while the emulsions of Fractions B and C each contained 5 per cent of these substances. The dose administered was in each case 0.6 cc. of the emulsion. No ill effects were observed to follow any of the administrations.

The average diameter of the tumors in the control animals on the 21st day after inoculation was 13.1 mm. The diameters of the tumors in the animals subsequently treated with Fractions A, B, and C were 11.3, 13.4, and 11.1 mm., respectively. Calling these initial diameters in each group 100, and referring each of the subsequent measurements to this unit of comparison, we obtained the following results.

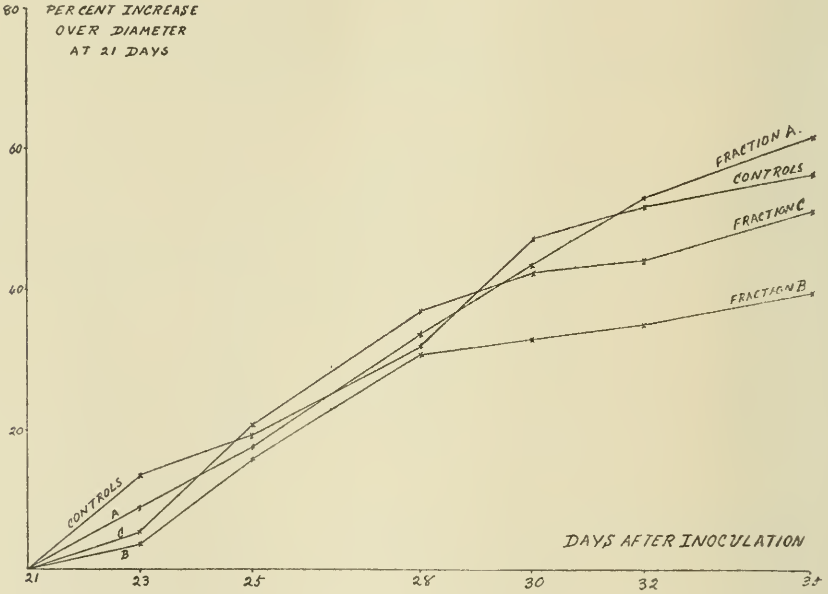
TABLE II.

Days after inoculation.	Diameter of tumors.			
	Controls.	Fraction A.	Fraction B.	Fraction C.
21	100.0	100.0	100.0	100.0
23	113.7	108.8	103.7	105.4
25	119.1	117.7	115.7	120.7
28	132.1	133.6	130.6	136.9
30	147.3	143.4	132.8	142.3
32	151.9	153.1	135.1	144.1
35	156.5	161.9	139.6	151.3

These results are depicted graphically in Text-fig. 2. It will be seen that neither Fraction A nor Fraction C exerted any significant action upon the growth of the tumors. Fraction B, however, caused a marked retardation of the growth of the primary tumors. It will be recollected that Fraction B, consisting as it does of substances soluble in alcohol and in ether, and precipitable by acetone, is composed almost entirely of lecithin. The retardation of tumor growth following its administration is therefore merely confirmatory of our previous finding[5] that administrations of lecithin markedly diminish the rate of growth of the Flexner-Jobling carcinoma in rats. The lecithin employed in our previous experiments, however, was prepared from yolks of eggs by extracting them with ether and adding acetone to this extract. It therefore probably consisted of a mixture

[5] Robertson, T. B., and Burnett, T. C., *Jour. Exper. Med.*, 1913, xvii, 344.

of approximately equal parts of lecithin and cephalin,[6] while the lecithin employed in the experiments just described, owing to the manner of its preparation (extraction of the tissue with alcohol) cannot have contained any admixture of cephalin. Evidently, therefore, lecithin itself is capable of causing the retardation observed in these and our previous experiments. Whether cephalin is capable of



TEXT-FIG. 2. The effect of hypodermic administrations of alcohol-soluble extractives from the anterior lobe of the pituitary body (other than tethelin) upon the growth of carcinomata. Note the retardation due to the lecithin fraction (Fraction B).

exerting a like retardation or whether it only acted in our previous experiments as an inert diluent of the lecithin remains to be ascertained at some future date.

In our previous experiments, moreover, the lecithin was administered by direct injection into the tumors. In the present experiments the lecithin was injected at a locality remote from the tumors.

[6] Stern, M., and Thierfelder, H., *Ztschr. f. physiol. Chem.*, 1907, liii, 370.

Evidently the retarding effect of lecithin is not attributable to a purely local action.

Since Fraction C did not exert an accelerative action upon the growth of the tumors, its content of free cholesterol was presumably small;[5] no analytical determinations have, however, been made.

On the 49th day the surviving animals (twenty-five of each group) were killed and the viscera examined for metastases. The control group and the groups treated with Fractions A and B, respectively, each yielded three animals, or 12 per cent, which had developed metastases. The group treated with Fraction C yielded five animals with metastases, or 20 per cent. It is possible that this slightly enhanced tendency to metastasize may have been attributable to the cholesterol content of this fraction.

CONCLUSIONS.

1. The hypodermic administration of tethelin increases markedly the rate of growth of the primary tumor and the tendency to form metastases in rats inoculated with carcinoma, in this, as in other respects, reproducing the action of the whole anterior lobe of the pituitary body.

2. Other alcohol-soluble extractives of the anterior lobe of the pituitary body, with the exception of the lecithin fraction, exert no appreciable effect upon the growth of carcinomata in rats.

3. The lecithin fraction, as in previously reported experiments in which we employed lecithin obtained from eggs, causes evident retardation of the growth of carcinomata in rats.

In conclusion we desire to express our indebtedness to Mr. Ralston B. Brown, Superintendent of the Oakland Meat and Packing Company, to whose cooperation we owe the supply of pituitary glands which has afforded us the opportunity of carrying out these investigations.

THE INTRAVENOUS INJECTION OF MAGNESIUM SULPHATE FOR ANESTHESIA IN ANIMALS.

By J. AUER, M.D., and S. J. MELTZER, M.D.

(*From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 10, 1916.)

The effect on animals of intravenous injections of magnesium sulphate was investigated by us from a general experimental point of view about ten years ago.[1] The use of this salt for practical purposes of anesthesia in human beings was first studied by Haubold and Meltzer by the intraspinal method.[2] About two years ago a combination of subminimal doses of magnesium sulphate intramuscularly and ether by intratracheal insufflation was found by the present writers[3] to be effective in animals, and by Peck and Meltzer and also Elsberg and Meltzer in human beings. The use of magnesium sulphate by intravenous injection was in general discouraged by one of us.[4] However, a series of experiments made by the present writers with intravenous injection of magnesium sulphate in cases of experimental tetanus,[5] and the meager but satisfactory experience which Kohn[6] and Straub[7] had with the employment of this method in cases of tetanus in human beings, induced us to take up the experimental study in animals of the employment of magnesium sulphate by intravenous injection for the purpose of producing anesthesia. This was done as a preliminary test for the admissibility of

[1] Meltzer, S. J., and Auer, J., *Am. Jour. Physiol.*, 1905–06, xv, 387.

[2] Haubold, H. A., and Meltzer, S. J., *Jour. Am. Med. Assn.*, 1906, xlvi, 647.

[3] Meltzer, S. J., and Auer, J., *Proc. Soc. Exper. Biol. and Med.*, 1912–13, x, 159; *Zentralbl. f. Physiol.*, 1913–14, xxvii, 632.

[4] Meltzer, S. J., *Berl. klin. Wchnschr.*, 1915, lii, 261.

[5] To be published later.

[6] Kohn, H., *Berl. klin. Wchnschr.*, 1915, lii, 89.

[7] Straub, W., *München. med. Wchnschr.*, 1915, lxii, 341.

studying the exclusive use of intravenous injections of magnesium sulphate as a means of producing, or at least inducing, anesthesia in human beings. We wish to record a few abbreviated protocols of these experiments.

EXPERIMENTAL.

The experiments were made on dogs. The left external jugular vein was exposed under local anesthesia by ethyl chloride, a cannula introduced, and magnesium sulphate in an $\frac{M}{4}$ solution injected through the cannula from a burette. The reaction of the animals was tested in various ways, as indicated in the protocols. When the respiration appeared to be shallow, pharyngeal insufflation[8] was employed either temporarily or throughout the entire experiment. In some instances intratracheal insufflation was given, especially for the purpose of testing the possibility of introducing the intratracheal tube without using any other anesthetic and while the animal was still breathing spontaneously. At the end of the experiment either a small quantity of a calcium chloride or sodium sulphate solution was injected, or no further injection was given.

Experiment 1.—Black and white female fox-terrier; weight 4,600 gm.

10.20. On electric warming pad at medium. Clip hair of neck; used ethyl chloride as local anesthetic for exposing and inserting cannula in external jugular vein.

10.45. Rectal temperature 38.8°C.

10.50. Start infusion of $\frac{M}{4}$ magnesium sulphate into jugular vein.

10.55. Operation completed.

11.00. 5 cc. Lid reflex prompt and strong.

11.04. 14 cc. Slightly restless.

11.05½. Lid reflex prompt; active expiration, of good strength.

11.08. 20.5 cc. Lid reflex prompt and strong. Respiration good, fairly rapid, active expiration.

11.11. 26 cc. Respiration fairly rapid; quiet; lid reflex prompt and strong.

11.13. 33 cc. Respiration slower, with active expiration; no sound. Heart slow.

11.15. Catheter F. 19 inserted into trachea with ease; start air insufflation.

11.17. 40.5 cc. Lid reflex slight.

---

[8] Meltzer, S. J., *Jour. Am. Med. Assn.*, 1913, lx, 1407; *Berl. klin. Wchnschr.*, 1915, lii, 425.

11.18. Very slight inspiration, slow rate; able to move head slightly.

11.20. 44.5 cc. Very shallow respiration on stopping insufflation.

11.22. 46.5 cc. Lid reflex fair.

11.23. Occasionally spontaneous, fair expirations during insufflation.

11.24. 50 cc. injected. Stop. Very slight spontaneous shallow respiration; slow rate. Lid reflex slight.

11.25. Injected through venous cannula 15 cc. $\frac{M}{8}$ calcium chloride into jugular vein. Respiration began promptly. Injection lasted about 1 minute. Suture wound after wiping with diluted tincture of iodine. Remove catheter. Animal trots away at once, slightly unsteady at first; wags tail, obeys command.

12.20. Runs about with ease.

2 days later, 10 a.m. Dog lively and active, jumps about, barks, behaves like a normal dog.

Next day. Good condition.

The intravenous injection of an $\frac{M}{4}$ solution of magnesium sulphate was given at a slow rate. After injecting 33 cc. in 23 minutes the intubation for intratracheal insufflation was performed with ease, while usually a great deal of ether has to be given to accomplish this purpose. A total of 50 cc. of magnesium was given in 34 minutes. The spontaneous respiration was then very shallow. After injecting 15 cc. of $\frac{M}{8}$ calcium chloride the respiration recovered promptly and the animal would execute satisfactory voluntary movements.

Experiment 2.—Black and white mongrel, male; weight 5,850 gm.

Preliminary preparations as in Experiment 1.

11.37. Start infusion of $\frac{M}{4}$ magnesium sulphate into jugular vein.

11.41. 9 cc.

11.44. 18 cc. Moves head, lid reflex strong. Barks sleepily.

11.46. 22 cc. Respiration more rapid, shallower; no reaction to pricking skin with needle; lid reflex strong.

11.50. 36 cc. Fair, slow respiration with active expiration. Lid reflex prompt. Occasional slight general motions. Heart slowed.

11.52. 39.5 cc. Respiration slow, moves head.

11.55. 46 cc. Very slow respiration, lid reflex fair; heart slow.

11.57. 50 cc. Respiration very slight; start pharyngeal insufflation. Pulse improved.

12.02. 54.5 cc. Stop insufflation to change stomach tube; no respiration seen.

12.04. 55.5 cc. Heart 24 to $\frac{1}{4}$ minute, small, regular, soft.

12.06. 56.5 cc. Breathes spontaneously; no reaction to pricking skin with needle.

12.09. 61 cc. Tracheal catheter inserted; slow spontaneous respiration, fair depth, some active expiration; lid reflex slight, moves head.

12.12. 64 cc. Legs relaxed; spontaneous respiration fair; lid reflex slight.

12.15. Pulse 27 to $\frac{1}{4}$, regular, strong (femoral); fair spontaneous respiration. Stop insufflation. 65.5 cc. injected. Respiration gradually improves and becomes good, deep. Respiration moderately slow; start air again.

12.16. Excellent respiration; no lid or corneal reflex. Tongue pink.

12.20. Respiration easy, more rapid; no lid or corneal reflex. 70 cc. injected.

12.22. Moves head; increase magnesium flow slightly.

12.23. 73 cc. Respiration slower, but good depth. Femoral pulse 22 to $\frac{1}{4}$, respiration good depth.

12.27. No reaction to pricking skin. 78.5 cc. Stop magnesium.

12.28. 8 cc. of $\frac{M}{8}$ calcium chloride into jugular vein. Respiration greatly improved and more rapid. Wound sutured. Placed on floor, holds head up; front legs spread, do not support body; licks jaws; moves tail on pressure. Pays attention to call and wags tail.

12.35. Able to walk about; tail erect.

2.10. Walks about normally.

Next day. Good condition.

The first 50 cc. injected in 20 minutes, about 2.5 cc. per minute, nearly completely abolished respiration. Pharyngeal insufflation was then started and exerted immediately a good effect. From 12.02 to 12.06 only 2 cc. were injected, equal to 0.5 cc. per minute; spontaneous respiration returned, but no reaction to pricking, and intubation of catheter was easily executed. Thereafter the rate of inflow was kept fairly low. Spontaneous respiration was continually present but was generally slow. There was no lid reflex and no reaction to pricking. After injecting about 78 cc. of magnesium in 50 minutes a quantity of 8 cc. of $\frac{M}{8}$ calcium chloride was injected; respiration improved at once. Voluntary movements, however, returned gradually.

Experiment 3.—Male; weight 7,500 gm.

Was fed previous to experiment. Preparation as in previous experiments.

2.27. Rectal temperature 38.7°. Femoral pulse 33 to $\frac{1}{4}$, regular.

2.33. Start $\frac{M}{4}$ magnesium sulphate into jugular vein.

2.37. 7 cc. Respiration deeper; swallows occasionally.

2.40. Pulse softer, 38 to $\frac{1}{4}$, regular; respiration more rapid with strong active expiration. 14 cc.

2.41. Vomited yellowish brown fluid.

2.43. Vomited large amount of yellow fluid with masses of meat.

2.44. 22 cc. Lid reflex prompt; occasional moderate struggle.

2.48. 32 cc. Moderate barks. Lid reflex strong.

2.49. 38.5 cc. Respiration chiefly expiratory. Tracheal catheter inserted; animal shows resistance. Catheter F. 21; constant air stream with remission. Lid reflex strong.

2.54. 44 cc. No reaction to pricking skin with needle.

2.55. 45 cc. Stop insufflation; practically only one inspiration and a number of weak abdominal contractions appeared during the intermission. Lid reflex strong. Able to move head moderately. Occasionally a sharp expiratory movement of abdomen. Started intratracheal insufflation again.

3.06. Remove tracheal catheter; start pharyngeal insufflation, tube in stomach.

3.10. 57 cc. Moves head vigorously; femoral pulse regular, soft.

3.13. 59.5 cc. Legs limp. Lid reflex good.

3.22. 73 cc. Open abdomen, rub peritoneum above liver; no motions.

3.23. 76 cc. Lid reflex fair.

3.26. 80 cc. Stop insufflation; no definite respiration, some expiratory contractions. Start insufflation. Abdomen closed.

3.31. 80 cc. No definite respiration on stopping insufflation. Good lid reflex. Pupils wide.

3.35. 90 cc. Stop magnesium. Lid reflex fair. Stop air; 3 slight respirations; start insufflation.

3.38. Slight, slow, shallow respiration on stopping pharyngeal insufflation. Femoral pulse 18 to $\frac{1}{4}$, regular, fair tension.

3.40. 10 cc. of $\frac{M}{8}$ calcium chloride into jugular vein. Deep, slow respirations begin.

3.43. Pulse 29 to $\frac{1}{4}$, regular, strong tension; no reaction to pricking skin with needle. Lid reflex strong; pupils wide. Neck wound closed. Placed on floor, attempts to get up.

3.44. Gets up after a few trials.

3.55. Lies on side; no response to pressure on toes; wags tail; tolerates probe in nose for a short time. When placed on feet walks away fairly steadily, then lies down again.

4.25. As before; no response to pressure on legs; lies on side usually. Raises head on call; walks when placed on feet. Lid reflex prompt; drinks some water. Killed later by chloroform.

This animal was fed about 3 hours before the experiment was started. After 14 cc. of the magnesium solution were injected (in 7 minutes) the animal vomited. This indicates the central action of magnesium sulphate. After 45 cc. were injected (in 22 minutes) there was practically no spontaneous respiration, although the lid reflex was strong and the animal was able to move its head. The failure of the respiration in this case was undoubtedly due to the in-

hibitory action of the magnesium sulphate upon the respiratory center, and not to a paralysis of the motor nerve endings of the respiratory muscles, which, as a rule, remain excitable longer than the other skeletal muscles. After 80 cc. of the magnesium solution (in 58 minutes) the abdomen was opened and the sensitive parts of the parietal peritoneum were rubbed without eliciting any reaction, although the lid reflex was still good. This animal received 90 cc. of the magnesium solution in 62 minutes. 3 minutes after stopping the injection the animal had only slow and shallow respirations. The injection of 10 cc. of $\frac{M}{8}$ calcium chloride deepened the respirations; it exerted also, fairly promptly, a favorable effect upon the general motility of the animal. But the return of reactions to a probe inserted into the nose and to other sensory stimuli was slow.

Experiment 4.—Wolf hound, female; weight 6,700 gm.

Preliminary preparations same as in Experiment 1.

2.42. Start $\frac{M}{4}$ magnesium sulphate into jugular vein.

2.43. 3 cc.

2.46. 10.5 cc. Lid reflex prompt, sustained. Respiration faster; pulse fuller, faster, 33 to $\frac{1}{4}$. Respiratory irregularity of pulse rhythm practically gone.

2.48. 16.5 cc. Respiration 18 to $\frac{1}{4}$, fair depth, slight active expiration.

2.50. 22 cc. Lid reflex prompt, but no longer sustained closure.

2.52. 28 cc. Respiration good, 14 to $\frac{1}{4}$; femoral pulse 30 to $\frac{1}{4}$, good volume and tension; no irregularity.

2.55. 36 cc. Respiration slower; pain sensation of skin abolished.

2.56. 39 cc. Respiration slower, but good depth; pulse irregular. Start pharyngeal insufflation. Stomach tube.

2.58. 46 cc. Lid reflex prompt but slight. Pupils wide.

3.00. 47 cc. Open abdomen and rub diaphragm; no motion of any kind.

3.02. Stop insufflation; very slight shallow respiration; start air; lid reflex a slight flick. Pulse 36 to $\frac{1}{4}$, regular, small volume and tension. Rub peritoneum; no sign of movement; legs limp.

3.07. 51 cc. Lid reflex slightly better; rubbing peritoneum causes no motion of any kind. No knee jerk.

3.08$\frac{1}{2}$ 52.5 cc. Stop insufflation; 16 very slight respirations per $\frac{1}{4}$. Rub diaphragm and parietal peritoneum: no motion. Legs limp. Lid reflex fairly sustained now (closure).

3.15. 56.5 cc. Femoral pulse 32 to $\frac{1}{4}$, regular, small, and soft.

3.17. 57 cc. Stop air; no respiration in 35 seconds. Start air. Lid reflex slight; pupils moderately contracted.

3.21. 58.5 cc. Rub peritoneum and diaphragm; slight motion of leg, but no other perceptible movement. Spontaneous respiration noticeable during insuffla-

tion. Stop air; 24 shallow respirations per $\frac{1}{4}$. Start air. Pulse 32 to $\frac{1}{4}$, fair volume and better tension now. Lid reflex prompt but weak.

3.25. 59.5 cc.

3.26. Moves leg slightly.

3.29. 61 cc. On rubbing peritoneum and diaphragm no motion, but later made vigorous movements with head and leg. (No apparent relation to stimulus; rubbing repeated; no motion.)

3.31. Some strong movements of head and legs. Lid reflex flick, not sustained. Stop air. Respiration spontaneous, 15 to $\frac{1}{4}$, good depth. Stop insufflation entirely.

3.33. 63 cc. of magnesium. Stop. Excellent respiration. Suture abdominal wound. Ligate jugular vein and suture wound in neck. No calcium chloride given.

3.36. Placed on floor; attempts to walk; hind legs spread; raises head and looks about.

3.43. Lying on side; placed on legs; walks about; lies down again shortly. Withdraws legs fairly promptly when pressed; walks away when tail is pressed; no sign of pain.

4.00. Walks about. Rectal temperature 36.4°; pulls away leg when toes are pressed.

4.38. Killed by chloroform.

During the first period of the experiment (about 18 minutes) the inflow of the magnesium solution occurred at a rate of about 2.9 cc. per minute. After the injection of 36 cc. (in 13 minutes) skin sensibility was abolished; the respiration, though slower, was good and there was even an occasional struggle. The pharyngeal insufflation was started before there was any necessity for it. After the injection of 47 cc. (in 18 minutes) the abdomen was opened and the peritoneum rubbed without any reaction. About this time, however, the spontaneous respiration was shallow, lid reflex slight, and the legs were limp. In the following half hour the rate of injection was considerably reduced—about 16 cc. in 33 minutes. The loss of sensibility lasted for about half an hour longer. The spontaneous respiration returned perceptibly sooner. Altogether 63 cc. of magnesium sulphate were injected in about 51 minutes. Then, when the magnesium sulphate injection was stopped and the insufflation discontinued, the spontaneous respiration immediately appeared to be excellent. No calcium chloride was given. After placing the animal on the floor, motility and sensibility returned fairly soon. The quan-

tity of magnesium given in this experiment was not large and the rate of injection slowed down considerably during the latter part of the experiment. The recovery here was prompt and without the aid of calcium chloride.

Experiment 5.—Bull terrier, female; weight 7,600 gm.

Preparation the same as in previous experiments.

Rectal temperature 40.2°. Femoral pulse 30 to $\frac{1}{4}$, small, regular, good tension. Respiration slow, 12 to $\frac{1}{2}$, with active expiration.

10.58. Start $\frac{M}{4}$ magnesium sulphate into jugular vein.

11.04. 17 cc. Lid reflex prompt but not sustained; pupils wide. Respiration less deep, slow. Swallows occasionally.

11.06. 25 cc. 14 respirations to $\frac{1}{2}$, good depth, moderate active expiration.

11.07. 29 cc. Lid reflex prompt and sustained. Quiet.

11.08. 35 cc. Pulse small, fairly soft, 38 to $\frac{1}{4}$, regular. Respiration good depth.

11.10. 43 cc. Respiration slow, less deep but still good, 7 to $\frac{1}{4}$. Slow magnesium inflow.

11.13. 50 cc. Respiration improved.

11.15. 53 cc. Magnesium inflow slowed. Lid reflex fairly prompt.

11.18. 56 cc. Open abdomen; rub peritoneum and diaphragm; no motion of any kind. Respiration good, more rapid than before.

11.20. 58.5 cc. Lid reflex slight; respiration rapid, 36 to $\frac{1}{4}$, next count 28 to $\frac{1}{4}$; legs relaxed. Expose left sciatic nerve; no motion at first, later moderate general movements.

11.25. 71 cc. Respiration excellent.

11.29. 86 cc. Respiration much shallower and slower. Start pharyngeal insufflation.

11.32. 98 cc. No lid reflex.

11.34. 102 cc. No definite respiration.

11.37. 104.5 cc. Stimulated left intact sciatic with Petzold inductorium. At coil distances of 200 and 120 mm. no reaction elicited. At 80 mm. respirations appeared during stimulation; left toes moved slightly; also weak general motions and movements of tail. Rub peritoneum and diaphragm; no response.

11.42. 107 cc. Stop pharyngeal insufflation; slight spontaneous respiration present; start pharyngeal insufflation; increase magnesium flow slightly. Limp. No lid reflex; pupils very wide.

11.47. Abdomen and thigh wound sutured. 116 cc. No lid reflex.

11.49. 117 cc. Stop magnesium. Spontaneous respiration very slight; pharyngeal insufflation necessary.

11.50. 60 cc. $\frac{M}{4}$ sodium sulphate into jugular vein. Respiration improved promptly. Stop pharyngeal insufflation.

11.53. Pulse good. Suture neck wound, lid reflex slight.

11.56. Placed on floor, holds head up for short time, then rests it on floor; cannot stand. Wags tail when called.

12.01. Pressure on tail and toes; moves head towards tail, draws away foot.

12.05. Lid reflex very slight, pupils wide. Able to get up but prefers to squat or lie down.

1.55. Walks about readily, keeping left hind leg lifted (left sciatic nerve had been exposed), no staggering; lid reflex prompt and sustained. Pupils well contracted. Urinated large amount; first time since injection.

4.45. Walks about easily when placed on feet (staid in one place since last note); no more urine passed. Killed by chloroform.

This dog had from the start a slow respiration although its temperature was higher than normal. During the first 10 minutes of the magnesium infusion 3.5 cc. per minute were injected, more than in any of the animals in previous experiments. There were no struggles. After injecting 56 cc. the abdomen was opened and the parietal peritoneum rubbed without any reaction, while the respiration was good and even more rapid than before. After 102 cc. no definite respirations were present, and after 104 cc. the motor nerve endings were affected. The inflow was then reduced—only 13 cc. in 12 minutes. Altogether 117 cc. were injected in 51 minutes. There was practically no spontaneous respiration when the infusion of the magnesium solution was discontinued. However, the respiration improved within 1 minute after the injection of 60 cc. of $\frac{M}{4}$ sodium sulphate. The general motor and sensory depression seemed also favorably affected by this injection.

Experiment 6.—Black male; weight 7,500 gm.

Preliminary preparation as in Experiment 1.

3.13. Start $\frac{M}{4}$ magnesium sulphate into jugular vein.

3.17. 12 cc. Respiration 8 to $\frac{1}{4}$. Good depth. Femoral pulse 29 to $\frac{1}{4}$, regular, good volume and tension.

3.18. 15 cc. Lid reflex prompt and strong, pupil moderately dilated. Dog quiet.

3.20. 25 cc. Good respiration, 12 to $\frac{1}{4}$, active expiration stronger. Femoral pulse 32 to $\frac{1}{4}$, regular, good tension. Barks.

3.22. 31 cc. Lid reflex prompt and sustained; pupils wider.

3.23. 34.5 cc. Pain abolished; opening of peritoneum; respiration easy, good depth and frequency. Abdomen relaxed.

3.25. 46 cc. Rub peritoneum and diaphragm; no movement. Lid reflex prompt and sustained; respiration slow, good depth, 11 to $\frac{1}{4}$.

3.27. 50 cc. Blood bright red. Slow magnesium inflow.

3.28. 51 cc. Rub peritoneum of diaphragm; no movement. Lid reflex weak.

3.30. Pulse 30 to $\frac{1}{4}$, regular, good volume and tension.

3.31. 53.5 cc. Slight knee jerk.

3.32. 55 cc. Moved legs; respiration faster and deeper. Rub peritoneum and diaphragm; no immediate effect, after a few seconds rapid respiration with moderate strength. Increase magnesium inflow.

3.34. 61 cc. Lid reflex a mere flick; rapid respiration with active expirations in short group, then easy respirations without active expirations.

3.39. Respiration slow, good depth with active expiration.

3.42. 85 cc. Blood a little darker; respiration shallower.

3.44. Start pharyngeal insufflation.

3.46. Pulse 20 to $\frac{1}{4}$, fair volume and tension, regular. No lid reflex. Rub peritoneum and diaphragm; no movement. Legs limp; no knee jerk.

3.52. No spontaneous respiration on stopping insufflation.

3.55. Pupils well dilated but not maximal; no lid reflex. 103 cc. Pulse small, 25 to $\frac{1}{4}$.

4.00. 104.5 cc. No lid reflex. Rub peritoneum; no movement; no knee jerk.

4.05. No spontaneous respiration on stopping insufflation; pulse weak. Start insufflation again.

4.07. No movement on rubbing peritoneum and diaphragm. 106 cc. Stop magnesium. No lid reflex. Injected 60 cc. $\frac{M}{4}$ sodium sulphate into jugular vein.

4.10. Femoral pulse 25 to $\frac{1}{4}$, small, regular, better tension. Rubbing peritoneum and diaphragm; no movement.

4.11. Stop insufflation; slow respiration, getting deeper; start insufflation again. No lid reflex; pupil wide.

4.13. Rub peritoneum; no movement. Legs limp; no knee jerk.

4.17. Suture abdomen and neck wound and stop insufflation. Pulse 27 to $\frac{1}{4}$, regular, good volume and tension; 4 respirations to $\frac{1}{4}$, good and deep, no active expiration; no lid reflex, pupils wide; 36.4°. Placed on floor; cannot stand, lies on side. Pain sensation fair; looks about and wags tail.

4.25. No lid or corneal reflex; wags tail when called; feeble knee jerk; on moderate pressure of toe pads, no movement. When lifted and placed on floor front legs bear body weight, but not the hind legs. Pupils widely dilated.

4.30. Respiration easy, good depth, 14 to $\frac{1}{2}$, no active expiration. Femoral pulse 29 to $\frac{1}{4}$, regular, good volume and tension. Lies on side, wags tail. Very slight lid reflex.

4.45. Sits up on haunches, but does not walk about; lid reflex fairly good. Passed small amount of urine.

5.00. Walks about, no weakness; lid reflex prompt and sustained; pupils still wide. Killed with chloroform.

This strong dog received in the first 10 minutes about 35 cc. of the magnesium solution. There was very little excitation, and at the

end of this period the skin of the abdomen could be incised to the peritoneum without any reaction. In the following 2 minutes 11 cc. were infused, and the sensitive parts of the parietal peritoneum were energetically rubbed without producing any reaction, while respiration was still good and the lid reflex prompt and sustained. The rate of injection was now reduced, and the spontaneous respiration kept up efficiently for some time. After 19 minutes during which time about 44 cc. were injected (a little less than 2.5 cc. per minute) pharyngeal insufflation was started. In the next 23 minutes only about 16 cc. were injected (about 0.7 cc. per minute). During this period there were no spontaneous respiration, no lid reflex, no knee jerk, and the legs were limp; finally the pulse became weaker. Altogether 106 cc. of the magnesium solution were injected in 54 minutes. At the end of the magnesium injection no calcium chloride was given, but, as in the previous experiment, 60 cc. of sodium sulphate in $\frac{}{4}$ solution were injected intravenously. The effect of the injection in this experiment, however, was in no way striking. The respiration did not improve at once and the insufflation had to be continued for about 10 minutes longer. The fact should be borne in mind that in this experiment the rate of injection of the magnesium sulphate during the first half of the infusion period was considerably greater than in any of the other experiments.

In addition to the foregoing experiments we wish to record briefly the exceptional course of one of the experiments. This dog had an irregular heart beat and its extremities were rigid before the experiment was begun. There was no spontaneous respiration after injecting 44 cc. of magnesium solution (in 14 minutes), while the peritoneum remained sensitive and the lid reflex active during most of the injection period. The animal received 72 cc. in 57 minutes. The pulse was small and often weak during the last half hour. At the end of the magnesium injection 10 cc. of $\frac{M}{8}$ calcium chloride were injected without restoring the spontaneous respirations. A few minutes later 5 cc. more of the calcium chloride brought on some weak respirations, but the heart stopped soon after and the animal died.

Here was a case in which calcium did not restore the respiration which had been abolished by magnesium; on the contrary, it was perhaps instrumental in accelerating cardiac death.

SUMMARY AND CONCLUSIONS.

These experiments justify the following general conclusions.

By the intravenous injection of $\frac{M}{4}$ magnesium sulphate into dogs at a certain rate, a stage can be reached where the abdominal walls are completely relaxed and when section of the abdomen and stimulation of sensitive parts of the parietal peritoneum do not produce pain or elicit any reaction of the animal. At the same time spontaneous respiration may still be maintained within normal limits and the lid reflex be fair or even normal. In this stage intratracheal intubation for artificial respiration can be easily accomplished. This stage may be attained in 12 to 14 minutes when the rate of injection is about 3 cc. per minute. When this stage is once attained the rate of injection should gradually be reduced, otherwise, sooner or later, spontaneous respiration will be abolished, and by a further maintenance of the rate of injection all the skeletal muscles may become paralyzed.

When the injection of magnesium is continued for a longer period, the paralytic effects of the magnesium injection will set in, even when administered at a slow rate.

The paralysis of the respiratory function is readily met by intrapharyngeal insufflation, which is easily executed even without training in this procedure, or by the method of intratracheal insufflation, if executed by one trained in its management.

When the respiration of the animal is accomplished by insufflation, the paralytic effect of the magnesium may be abolished fairly rapidly by an intravenous injection of about 10 cc. of an $\frac{M}{8}$ calcium chloride solution; or it may disappear slowly, after the infusion of the magnesium solution is discontinued for some time. The latter mode of disappearance may be favorably accelerated by an intravenous infusion of 60 to 100 cc. of an $\frac{M}{4}$ solution of sodium sulphate.

The production of anesthesia by intravenous injection of magnesium sulphate should not be undertaken unless an apparatus for intrapharyngeal insufflation is at hand, because in exceptional cases the disappearance of spontaneous respiration may be one of the earliest consequences of the magnesium injection.

The injection of calcium chloride should not be employed in cases in which the subject shows cardiac insufficiency. In such instances, moreover, injections of magnesium should not be used for the purpose of anesthesia; at least not until greater experience has been acquired in the employment of this method.

AN EXPERIMENTAL STUDY OF THE ADDITIVE AND ANTAGONISTIC ACTIONS OF SODIUM OXALATE, AND SALTS OF MAGNESIUM AND CALCIUM IN THE RABBIT.

By F. L. GATES, M.D., and S. J. MELTZER, M.D.

(*From the Department of Physiology and Pharmacology of the Rockefeller Institute for Medical Research.*)

Plate 95.

(Received for publication, March 4, 1916.)

INTRODUCTION

On the basis of the hypothesis that magnesium favors inhibition of the various functions of the nervous system, Meltzer and Auer studied extensively in this laboratory the action of magnesium salts upon various animals. In injecting magnesium sulphate subcutaneously,[1] they found that a certain dose, which varies with the species of animals, is capable of producing profound anesthesia and paralysis from which the animal recovers. For rabbits this dose amounts to about 1.5 gm. of magnesium sulphate ($MgSO_4 + 7\ H_2O$) administered in a molecular solution. Larger doses cause the death of the animal, as a rule, by respiratory paralysis. With an effective but non-fatal dose in subcutaneous injections the development of the depressing, inhibitory effect is gradual and fairly slow. When the maximum is reached, the turn for the recovery sets in soon; there is practically no real plateau to the inhibitory curve. The descending limb of this curve—the recovery—is steeper than the ascending one. When a magnesium salt is injected intramuscularly, the inhibitory as well as the fatal effects set in more promptly and with smaller doses.

In the course of their studies, Meltzer and Auer[2] found that calcium, which is chemically closely related to magnesium, is biologically appar-

[1] Meltzer, S. J., and Auer, J., *Am. Jour. Physiol.*, 1905, xiv, 366.

[2] Meltzer, S. J., and Auer, J., *Am. Jour. Physiol.*, 1908, xxi, 400.

ently the antagonist of the latter. When calcium is injected intravenously, shortly before or immediately after the respiration stops, into an animal which has received a fatal dose of magnesium, the animal will recover in less than a minute, provided, of course, that the circulation is still effective during the calcium injection. This biological antagonism is a remarkable fact and can be made the basis of many problems worth investigating. So far, at least, it is not known that between calcium and magnesium salts which have the same anion a chemical antagonism exists; no precipitation, for instance, occurs *in vitro* when a solution of magnesium chloride is mixed with a solution of calcium chloride. Calcium chloride is nevertheless strikingly antagonistic to magnesium chloride as far as the life of animals and plants is concerned.

What effect would the deprivation of the animal body of some of its calcium have upon the behavior of the animal? There are a number of acids and salts which precipitate calcium compounds *in vitro*. Will the administration of these calcium-precipitating compounds, let us say oxalic acid or oxalates in general, bring out symptoms indicating an increase of magnesium action? By precipitating calcium within the body a certain amount of unantagonized magnesium would be set free. Would this fact become manifest by the appearance of inhibitory and paralytic phenomena? The symptoms of oxalate poisoning do not speak for it; in general they possess rather the opposite character: excitation, tremor, and convulsions. But the amount of magnesium thus set free and the inhibition which it may be capable of exerting, might under these circumstances be too small to play a perceptible part, in the presence of the violent opposite symptoms which are brought out by another exciting factor of the oxalate. Could, however, the depressing component of the calcium-precipitating oxalate be brought out by a simultaneous administration of a subminimal dose of a magnesium salt? This was the problem which we tried to solve experimentally.

While we were at work on this problem, Schütz[3] published a brief preliminary communication in which he says that the susceptibility to magnesium injections could be increased occasionally, but not

[3] Schütz, J., *Wien. klin. Wchnschr.*, 1913, xxvi, 745.

constantly by sodium oxalate. A few months later Starkenstein,[4] with whose work we were not familiar until after we had given a preliminary communication of our results,[5] stated in a preliminary report that he found "like Schütz that the addition of oxalates constantly gave a visible increase of the magnesium narcosis."

We shall describe briefly our experiments bearing upon the problem under discussion and the conclusions to which they point.

EXPERIMENTAL PART.

We experimented exclusively on rabbits. Magnesium sulphate in M solution ($MgSO_4 + 7\ H_2O$) and sodium oxalate in 3 per cent solution[6] were injected separately and practically simultaneously, either into the lumbar muscles on opposite sides of the spine, or subcutaneously into each flank, the injection being usually followed by brief massage. All doses were estimated and are here reported in gm. of the salt per kilo of body weight. Most of the experiments were performed on a series of three animals, two serving as controls and receiving subtoxic doses of either magnesium sulphate or sodium oxalate alone. The experimental animal received the same dose of both salts.

Intramuscular Injections.

An abbreviated typical protocol follows.

Experiment I.

Rabbit A.—Oct. 2, 1913. Magnesium sulphate alone. Grey female. Weight 1,550 gm.

11.10 Right lumbar muscles: magnesium sulphate M, 4.3 cc. = 0.7 gm. per kilo of body weight.

11.23. Lying down, head up, breathing rapidly.

---

[4] Starkenstein, E., *Wien. klin. Wchnschr.*, 1913, xxvi, 1235.

[5] Gates, F. L., and Meltzer, S. J., *Zentralbl. f. Physiol.*, 1913–14, xxvii, 1169. Starkenstein, E., *Zentralbl. f. Physiol.*, 1914, xxviii, 63; *Arch. f. exper. Path. u. Pharm.*, 1914, lxxvii, 45.

[6] Merck's reagent, "Sörensen's oxalate." Impure oxalates are not soluble to 3 per cent.

11.32. Can be placed on side.

11.34. Moves eyes and head when approached.

11.40. When tail is pressed, raises head and turns on belly. Moves head and looks around.

11.57. Hops when tail is pressed.

12.13. Hops around voluntarily, watching other rabbits. Remains well.

Rabbit B.—Oct. 2, 1913. Magnesium sulphate and sodium oxalate. Grey male. Weight 1,720 gm.

11.13. Right lumbar muscles: magnesium sulphate M, 4.8 cc. = 0.7 gm. per kilo. Left lumbar muscles: sodium oxalate 3 per cent, 5.6 cc. = 0.10 gm. per kilo.

11.24. Lying prone, with head on floor. When pushed over on side, lies passive. Respiration full; 17 in ¼ min. No response to pressure on tail; lid reflex good.

11.38. Same position. Lid reflex hardly perceptible.

12.01. Respiration slow and deep; 13 in ¼ min.

12.12. No response to stimuli, no lid reflex.

12.37. Turns over and lies on belly. Head sinks to floor and is raised at intervals. No response to pressure on tail.

1.08. Crouching in a corner. Recovers.

Rabbit C.—Oct. 2, 1913. Sodium oxalate alone. Grey male. Weight 1,480 gm.

11.21. Left lumbar muscles: sodium oxalate 3 per cent, 4.88 cc. = 0.1 gm. per kilo of body weight.

11.28. Sitting up, alert, changes position frequently. Starts suddenly without apparent cause.

11.43. Active, hops around, laps water, licks site of injection.

12.14. Has been behaving normally. Does not remain in one position long. No further effects noted.

The contrast in the behavior of the controls and the experimental animal is striking. The control animals were but little affected: this dose of sodium oxalate produced only trivial symptoms in Rabbit C, and the magnesium animal, Rabbit A, while weak and stupid, was at no time paralyzed or anesthetic. The third rabbit, however, ten minutes after the injections, was deeply anesthetized and remained passive and insensible for an hour. Two points are of particular interest: (1) In spite of the depth and long duration of the narcosis the respiration continued of good volume and rate and the animal was at no time in danger. (2) The animal regained power of voluntary movement before the return of sensibility to painful stimuli. A series of experiments with similar doses is given in Table I.

TABLE I.

Magnesium Sulphate and Sodium Oxalate, Intramuscularly.

No. of experiments.	Dose per kilo of body weight.		Average duration of deep inhibition. Animal relaxed on side.	Died.	Remarks.
	Magnesium sulphate.	Sodium oxalate.			
	gm.	*gm.*	*min.*		
6	0.7	0.10	89	0	
6	0.7	—	10	0	Only two out of six relaxed at all.
4	—	0.10	0	0	Visible effect questionable.

Subcutaneous Injections.

When the injections were made subcutaneously, somewhat larger doses had to be employed. A typical protocol of an experiment follows.

Experiment II.

Rabbit A.—Oct. 9, 1913. Magnesium sulphate alone. Slate colored female. Weight 2,030 gm.

10.16. Left flank, subcutaneously: magnesium sulphate M, 6.5 cc. = 0.8 gm. per kilo. Massage for 20 seconds.

10.31. Lying down, head and ears erect, breathing rapidly.

10.47. When disturbed hops away clumsily.

11.15. Crouching quietly, head up, ears flat on back. Respiration good.

11.50. Raises head to sniff at nearby objects.

12.20. Sitting up, washing paws. Remains well.

Rabbit B.—Oct. 9, 1913. Magnesium sulphate and sodium oxalate. Grey male. Weight 1,755 gm.

10.18. Right flank, subcutaneously: magnesium sulphate M, 5.6 = 0.8 gm. per kilo. Left flank, subcutaneously: sodium oxalate 3 per cent, 8.75 cc. = 0.15 gm. per kilo. Both sides massaged for 20 seconds.

10.53. Sitting up naturally.

11.04. Lying with chin on floor. Respiration slower and deep; 21 in $\frac{1}{4}$ min.

11.13. Placed passively on side without a struggle. Respiration 18 in $\frac{1}{4}$ min.

11.37. No response to pressing tail. Respiration shallow.

12.50. Trace of lid reflex. No response to pressing tail. Respiration of fair depth; 14 in $\frac{1}{4}$ min.

2.35. No lid reflex. No response to pressing tail.

3.52. Animal lying as before. No response to stimuli. Breathing entirely abdominal, of fair depth; 14 in $\frac{1}{4}$ min.

4.28. Does not resist handling. Voluntarily moves head, tail, and legs slightly. Observation discontinued.

Oct. 10, 1913. 9.15. Sitting up in cage. Rather quiet.

Rabbit C.—Oct. 9, 1913. Sodium oxalate alone. Grey female. Weight 1,510 gm.

10.22. Left flank, subcutaneously: sodium oxalate 3 per cent, 7.5 cc. = 0.15 gm. per kilo. Massage for 20 seconds.

10.43. Hops around licking the floor and sniffing at objects.

10.53. Sitting up, behaving normally.

11.14. Hops off actively when approached. No effects noted from injection.

Here again neither the oxalate nor the magnesium alone was effective. Together they produced a profound depression with a period of anesthesia and paralysis lasting more than four hours, followed by a gradual complete recovery.

Table II summarizes experiments with subcutaneous injections.

TABLE II.

Magnesium Sulphate and Sodium Oxalate, Subcutaneously.

No. of experiments.	Dose per kilo of body weight.		Average duration of deep inhibition. Animal relaxed on side.	Died.	Remarks.
	Magnesium sulphate.	Sodium oxalate.			
	gm.	*gm.*	*min.*		
8	0.8	0.15	123 +++	1	In four animals anesthesia extended into the night following. One died next day without recovery.
8	0.8	—	0	0	Practically no effect. Drowsiness in four cases.
8	—	0.15	0	0	No effects observable.

The cited protocols and the two tables illustrate the results obtained in these series of experiments. With the exception of two failures at the beginning, before the proper relation of dosage was determined, the experimental animal in every instance was definitely more deeply affected than the controls. The differences between the various experiments were only of degree, and depended upon the relation of the dose employed and the mode of administration, whether subcutaneous or intramuscular. With proper dose the

contrasts were striking and constant; while the controls were hardly visibly affected, the experimental animals were deeply anesthetized and paralyzed, the character of this inhibition being in general similar to that caused by large effective doses of magnesium alone.

In the following particulars the depression of the animals which received sodium oxalate and magnesium sulphate seemed to differ from that of animals which received magnesium alone. (1) The period of anesthesia and paralysis is a fairly long one, especially after subcutaneous injections, when the state of inhibition may last even 4 hours and longer; whereas after an effective sublethal dose of magnesium alone the entire state of depression is of a comparatively short duration. (2) In animals which receive oxalate and magnesium the deepest stage of anesthesia and paralysis tends to become stationary and is of long duration—the inhibitory curve has a long plateau—and the recovery takes place gradually; whereas with magnesium alone the inhibitory curve has hardly any plateau, and the animal after reaching the acme of anesthesia and paralysis either recovers quite rapidly or the depression leads to death by respiratory paralysis.

The increase of depression following the injection of subminimal doses of sodium oxalate and magnesium sulphate which was definitely established in these experiments cannot be considered simply as a summation of two similar effects. The symptoms brought on by oxalates are entirely dissimilar to those of magnesium inhibition. In our experiments the symptoms which follow the injections of sodium oxalate in toxic doses exhibit the character of excitation; anxiety, restlessness, hypersensitiveness, and tonic and clonic convulsions, which finally lead up to asphyxia and to a fatal termination. In such subminimal doses as we have employed, the toxic symptoms, if there were any, consisted at most in excitation and increased alertness; but there was never any manifest depression. It seems, therefore, that the strikingly depressing effect which the addition of a practically non-toxic dose of sodium oxalate to a subminimal dose of magnesium produces, must be ascribed to the ability of the oxalate to precipitate calcium from the body fluids and thus eliminate an element which biologically is antagonistic to magnesium.

The Action upon the Motor Nerve Endings.

Among the general effects of magnesium salts their depressing action upon the motor nerve endings stands out prominently. In minimal effective doses these salts reduce and in larger doses they completely abolish the conductivity of the nerve endings. In a series of experiments we have studied directly the combined action of sodium oxalate and magnesium sulphate upon this intermediary link between nerve and muscle. The sciatic nerve was cut under ether, the animal permitted to recover completely, and then the motor reactions of foot and toes to faradic stimulations of the peripheral end of the sciatic nerve were studied under the influence of the salts under discussion. Seventeen experiments were made upon rabbits. In fifteen there were two rabbits to each experiment, one an experimental animal and one a control. The experimental animals received subcutaneous injections of 0.6 to 0.8 gm. of magnesium sulphate in one side and 0.15 to 0.2 gm. of sodium oxalate. The fifteen control animals received injections of 0.6 to 0.8 gm. of magnesium sulphate alone. Two rabbits received injections of 0.15 and 0.2 gm. of sodium oxalate alone. For faradic stimulations a Porter induction coil, armed with one Daniell cell, was used. The cut sciatic nerve was stimulated before and at various intervals after the injection of the salt solutions, and the degree of the reactions to the various strengths of stimuli was noted. The results obtained in the experimental and control animals were compared and brought into relation with the general condition of the respective animals.

In both the sodium oxalate animals stimulation of the sciatic nerve before and at various times after the injection gave prompt reactions; strong tetanic flexion of the foot and abduction of the toes.

In eight of the magnesium controls stimulation of the sciatic nerve gave normal responses at the various periods after the injection. In the seven other controls there were slight degrees of reduction in the response to the stimulations; the reaction was less prompt, the extent of the contractions was lessened, or the distance of the secondary coil, in order to be effective, had to be shortened.

Of the experimental animals, in thirteen the conductivity of the peripheral nerve endings was definitely more deeply affected than in

their controls. In some cases the conductivity was so depressed that at the time when the general narcosis was at its height no response could be obtained from the stimulation of the sciatic nerve even with a 40 mm. coil distance. In two of the experimental animals the reduction in the response to stimulation of the sciatic nerve was not greater than that of their controls, although the general signs of anesthesia in the experimental animal were quite deep.

The depressing effect upon the motor nerve endings never outlasted the central effects, while there were cases in which the loss of sensation still continued after the motility seemed to be normal again.

The Antagonistic Action of Calcium.

Calcium, as stated in the introduction, is biologically antagonistic to magnesium, and our present experimental results led us to the conclusion that the increase of the depressive action of subminimal doses of magnesium by the addition of a subtoxic dose of sodium oxalate was due to the calcium-precipitating property of this salt. On the other hand, we found that the anesthesia and paralysis produced by a combination of subminimal doses of the two salts was of much longer duration than the same condition produced by an effective dose of magnesium sulphate alone. The question presented itself: Would calcium cause a recovery from the profound long-lasting state of depression caused by the combined action of the two salts, and especially would the recovery be as prompt and as rapid as in cases of magnesium anesthesia? We made a large number of experiments, but our results may be presented in the following single sentence: The antagonistic action of calcium is just as striking and prompt in the prolonged anesthesia brought about by the combination of oxalate and magnesium as it is in the anesthesia produced by magnesium alone. The following protocol is typical for all experiments in this series, and the photographs (Figs. 1 and 2) taken of this experiment are a good illustration of the results.

Experiment III.

Rabbit I.—Mar. 9, 1914. Magnesium sulphate alone. Grey and white male. Weight 1,860 gm.

1.58. Right back, subcutaneously: magnesium sulphate M, 5.9 cc. = 0.8 gm. per kilo. Massage for 1 min.

2.16. Sits quietly in corner of box, or lies down.

2.23. Crouching on forepaws, head and ears up. Respiration fair volume, slow; 19 in ¼ min.

2.32. Lying at full length, head and ears up. Backs up into sitting posture; rather heavy and quiet.

2.47. Crouching quietly in corner of box. Respiration full volume; 14 in ¼ min.

3.07. Photographed (Fig. 1).

3.16. Photographed (Fig. 2).

3.45. Behaving normally and has shown no further effects. Remains well.

Rabbit II.—Mar. 9, 1914. Magnesium sulphate and sodium oxalate. Black and white female. Weight 1,540 gm.

2.01. Right back, subcutaneously: magnesium sulphate M, 4.9 cc. = 0.8 gm. per kilo.

2.03. Left back, subcutaneously: sodium oxalate 3 per cent, 7.7 cc. = 0.15 gm. per kilo. Massage both sides for 1 min.

2.10. Has defecated. Respiration rapid and rather deep. Restless, changes position often.

2.13. Hind legs dragged a little in walking.

2.21. Crouching, head up, ears back, breathing rapidly; 68 in ¼ min.

2.35. Lying full length, eyes half closed, ears back, chin on floor. Flanks relaxed and bulging. Respiration 50 in ¼ min.

2.49. Lying partly on side, relaxed, head flat on floor. Mere trace of lid reflex. Moves head slightly when tail is touched.

3.05. Placed passively on back, feet in air. Remains there relaxed.

3.07. Photographed with controls (Fig. 1).

3.15. Same condition. Given 8 cc. calcium chloride 0.125 M through left ear vein. Respiration deepens during injection, and before it is completed animal turns over and sits up.

3.16. Photograph taken within 1 minute of injection (Fig. 2).

3.45. Crouching quietly. Hair erect. Hops off actively when disturbed. Then sits up with head and ears up. Remains well.

Rabbit III.—Mar. 9, 1914. Sodium oxalate alone. White female. Weight 1,620 gm.

2.06. Left back, subcutaneously: sodium oxalate 3 per cent, 8.1 cc. = 0.15 gm. per kilo. Massage for 1 min.

2.11. Hind legs dragged a little at times. Rather restless.

2.30. Sitting up or hopping around naturally. Head and ears up. Not restless or anxious. Respiration 42 in ¼ min.

2.48. Behaving normally. Sitting up, quiet. Respiration 35 in ¼ min.

3.07. Photographed (Fig. 1).

3.16. Photographed (Fig. 2).

3.45. Has shown no further effects.

Figs. 1 and 2 illustrate, in the first place, the anesthesia and paralysis produced by the combination of subminimal doses of magnesium and oxalate. They show, further, in a striking way, the antagonistic action of intravenous injection of calcium; it is in all respects similar to the action of calcium in anesthesia by magnesium alone. The respiration becomes deeper and more rapid immediately after beginning the injection, and the return of muscle tone and motor activity can be felt under the hand. Within a minute after the beginning of the injection, often indeed before all of the 8 or 10 cc. of solution is given, the animal draws up its legs, raises its head, turns over and scrambles into a sitting posture, and becomes alert and inquisitive. After an interval the rabbit may gradually sink back into narcosis, and can be restored again by calcium. Occasionally, if too much magnesium and oxalate have been given, a third injection may still be needed and given with success. However, under such circumstances, repeated injections of calcium might finally prove fatal to the animal.

The experiments, showing the depressing effect of magnesium and the antagonistic action of calcium to this depression, are, as we had occasion to learn, frequently demonstrated in many European Universities in lectures on pharmacology or physiology. When magnesium alone is used, the period of the greatest depression is of short duration and the demonstration may either be unconvincing, when the animal is not yet sufficiently narcotized, or it may be a failure, when the calcium injected is administered too late. The anesthesia and paralysis brought about by a combination of sodium oxalate and magnesium sulphate is, as we have seen above, of comparatively long duration. It is therefore a more appropriate method for purposes of demonstration. The animal may receive its double injection 40 to 50 minutes before the time set for the demonstration. If the proper doses are given and the proper procedure is followed out, there is no danger that the animal will not be in deep anesthesia, or that it will die too soon, before the antagonistic effect of the calcium can be shown.

SUMMARY.

The foregoing experiments establish firmly the following facts.

Subcutaneous or intramuscular injections of sodium oxalate in subtoxic doses, when administered to an animal which received a subminimal dose of magnesium sulphate, produce profound anesthesia and paralysis of long duration, although the usual effects of sodium oxalate alone are of a stimulating character. This fact is, in general, in harmony with the results reported by Starkenstein who, however, seems to have used the combination of the two salts in one solution; namely, that of magnesium oxalate.

The combined injections of subminimal doses of sodium oxalate and magnesium sulphate produce a strong reduction, or even, at times, a complete abolition of the conductivity of the motor nerve endings.

An intravenous injection of calcium salts brings on a recovery from the profound and prolonged effects of the combined action of sodium oxalate and magnesium sulphate, which is as prompt as is observed in experiments in which effective doses of magnesium alone were given. This fact is the more noteworthy, since depressions of long duration produced by prolonged continuous injections of magnesium solutions alone do not respond very promptly and effectively to calcium injections.

As will be recalled, the starting point for our investigation was the hypothesis that substances which are capable of precipitating calcium —a biological antagonist of magnesium—ought to be capable of increasing the depressive effect of magnesium. Our experiments proved that this assumption was correct. This would seem, therefore, to justify the interpretation that the augmenting action of sodium oxalate has its cause in the ability of the latter to precipitate calcium and thus increase within the body the amount of unantagonized magnesium. However, we wish to state expressly that this view is, for the present, still no more than a hypothesis and does not exclude other possible interpretations of our facts. As we pointed out it speaks against this hypothesis that oxalates do not produce phenomena of depression; the toxic symptoms produced by oxalates exhibit distinctly signs of increased and not of decreased irritability.

EXPLANATION OF PLATE 95.

Fig. 1. Rabbit III. Sodium oxalate 3 per cent, 0.15 gm. per kilo. Alert, ears erect. (Caught by instantaneous exposure.) Rabbit II. Sodium oxalate 3 per cent, 0.15 gm. per kilo. Magnesium sulphate M, 0.8 gm. per kilo. Deeply anesthetized and quite relaxed. Rabbit I. Magnesium sulphate M, 0.8 gm. per kilo. Crouches quietly as placed. Ears back.

Fig. 2. Rabbits III and I as before. Rabbit II within a minute has received 8 cc. of calcium chloride 0.125 M into the marginal ear vein (note clip). Alert and sensitive; right paw blurred from movement.

THE DISTRIBUTION OF TRYPAN-RED TO THE TISSUES AND VESSELS OF THE EYE AS INFLUENCED BY CONGESTION AND EARLY INFLAMMATION.

By PAUL A. LEWIS, M.D.

(*From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.*)

(Received for publication, February 24, 1916.)

It is the purpose of this paper to present certain simple observations which have a bearing on the manner in which the constituents of the aqueous humor are secreted into and eliminated from the anterior chamber of the eye. It will be shown that external influences may affect profoundly the ease with which abnormal constituents appear in the anterior chamber fluid. The observations have some bearing on general problems of pathology and pharmacology which will be pointed out. And lastly there will be included some observations on the diffusion of substances into and from the cornea.

All the observations have been made on the rabbit's eye. The azo-dye trypan-red as furnished by Grübler has been the substance chiefly employed for testing the permeability. This dye has been dissolved in normal saline solution to the amount of one-quarter of 1 per cent. 100 cc. of this solution may be warmed to body temperature and injected slowly into a full grown rabbit by the intravenous method without causing the animal any immediate distress and with no appreciable evidence of toxicity later. Under these circumstances by the time the injection is finished the skin and mucous membranes of the entire body are stained red. The intensity of the stain in the tissues increases for a number of hours to a maximum which is maintained without appreciable change for a number of days, and which then gradually fades out over a period of weeks and months. Trypan-blue and other azo-dyes of similar physiological activity may be used in the same general way.

Trypan-red is a colloidal substance; that is, its watery solutions do not diffuse through parchment paper. When, therefore, the dye leaves the blood vessels to appear in the tissues, the lymphatic spaces, lachrymal secretions, or the urine, its passage is in itself evidence that the interposed tissue surfaces are not perfect dialyzing membranes. They are either leaky mechanically to substances of certain physical constitution, or they exert a selective action which permits some colloids to pass while retaining others.

If the normal eyes are observed at any time after the injection of the dye in the manner described it will be seen that the sclera shares the stain of the skin with greater or less intensity. No stain can be detected in the cornea or anterior chamber fluid by inspection of the eye. If, however, after a number of hours the aqueous humor be withdrawn it will be found to have a barely appreciable pink color. If at the end of a week or 10 days the animal is killed, the eye removed, and the cornea dissected free, it also will be seen to be stained very faintly although definitely. If the blood be withdrawn immediately after the injection, the serum is found intensely stained with the dye. As the tissue stain increases in intensity, the stain disappears gradually from the blood to a minimum point which is probably long maintained.

Observations on the Aqueous Humor.

If within a few minutes after finishing the intravenous injection one eye is cocainized and the anterior chamber fluid withdrawn, a colorless fluid is obtained. As the chamber refills, which it does in the course of a short time, the reformed fluid is stained intensely. The rapidity with which a stained fluid appears in the anterior chamber following such a tap varies considerably in different rabbits. It is also possible so to alter the physiological condition of the eye that the dye when injected intravenously will quickly appear in the anterior chamber without the preliminary tapping.

With the facts above outlined as a basis for work, experiments have been carried out which throw some light on two distinct questions; namely, the place from which certain abnormal constituents may be secreted into the anterior chamber of the eye, and secondly the

relation between the different constituents of an altered anterior chamber fluid in the time of their appearance.

There is now a general agreement that the normal anterior chamber fluid is furnished from the vascularized ciliary body behind the iris, reaching the anterior chamber through the pupil. No experiment entirely free from objection has ever been devised either to prove or disprove this. The experiments in proof of the proposition have involved the withdrawal of fluid from the eye, a procedure which at once disturbs the relationships to an important degree. The experiments of Ehrlich[1] with fluorescin were interpreted by him to signify that the anterior chamber fluid was secreted from particular regions of the anterior surface of the iris. Ehrlich's experiments, done many years ago in part, show that fluorescin appears in the anterior chamber fluid from regions anterior to the iris. It should be recognized, however, that in interpreting such an experiment the fluid and the various other elements of either a normal or an altered aqueous humor may originate in different places. Those portions of Ehrlich's experiments which seemed to him to show that there were definite currents in the aqueous humor flowing from the sides toward the center, meeting on a vertical line in the midregion of the chamber in such a way as to form a swirl where the two streams meet, are susceptible of no explanation in the light of the conditions, as displayed by our work with trypan-red.

When the aqueous humor is withdrawn the pupil usually contracts more or less. Almost immediately the chamber begins to fill again with fluid. When the tap has followed shortly after the intravenous injection of trypan-red, the reformed fluid, as has been said before, is colored with the dye. The color comes for the most part through the pupil from behind. Occasionally it can be seen that the dye makes its appearance in considerable concentration on the anterior surface of the iris away from the pupilary margin before any color has appeared through the pupil.

If eserine is dropped in one eye in sufficient quantity to contract the pupil as far as possible, and if then the dye is injected intravenously, even though no aqueous humor is withdrawn the fluid will

[1] Ehrlich, P., Ueber provocirte Fluorescenzerscheinungen am Auge, *Deutsch. med. Wchnschr.*, 1882, viii, 21.

frequently become colored. Here again the color always appears from behind the pupil, but it may also appear on the anterior surface of the iris independently.

Neither cocaine nor atropine causes the appearance of color in the untapped eye. Atropine when used in such a way as to dilate the pupil fully before the eye is tapped prevents any appearance of dye on the anterior surface of the iris.

If one eye of a rabbit is cocainized and the cornea inoculated with a living culture of the tubercle bacillus, a progressive lesion results, differing in character with the particular culture used and the amount inoculated. The characteristics of this lesion we have described in detail elsewhere.[2] 24 hours after the inoculation there is usually an intense congestion of the conjunctiva, the iris and the ciliary body being more or less congested. This congestion tends to subside by the 2nd day but does not, as a rule, entirely disappear.

24 hours or more after such an inoculation, if the animal is injected with the dye intravenously as above described, the anterior chamber fluid of the inoculated, untapped eye will always become colored. Here again most of the color appears through the pupil, but in many instances it also appears earlier and independently on the anterior surface of the iris.

In each of these instances, the tapped eye, eserinated eye, or the tubercular eye, whenever the color appears on the anterior surface of the iris, it seems to be associated with definite areas of congestion. It would be interesting to know whether it is impossible to have the color appear abnormally in the absence of such a congestion. We have, with this in mind made some experiments with abrin. This poison, as is well known, causes an intense inflammation of the conjunctiva when it is dropped in the eye. The inflammation is characterized by a well marked edema and congestion and develops slowly through a stage which is, generally speaking, one of edema, to a stage which is more predominantly congestive. In the various stages of this inflammation trypan-red or trypan-blue applied intravenously appears in the anterior chamber, and in the edematous conjunctiva with unusual rapidity. We have never had a result, however, which

[2] Lewis, P. A., and Montgomery, C. M., *Jour. Exper. Med.*, 1914, xx, 269.

enables us to think that the dye can appear in abnormal amount or situation as a consequence of an edematous condition alone and in the entire absence of congestion.

To sum up this portion of our remarks we may say that under conditions in which the eye is slightly congested certain dyes injected intravenously may appear in abnormally large amount and with unusual rapidity in the aqueous humor. The largest amount of the dye comes into the aqueous humor from behind the iris, but appreciable amounts frequently come from the anterior surface of the iris. In these instances the iris has always shown a local congestion in the region in which the dye has appeared.

It is a well known fact that if the aqueous humor be withdrawn the fluid which refills the anterior chamber differs from that first withdrawn in that it coagulates spontaneously. We have tested the relationship between the appearance in the fluid of the factors controlling the coagulation and the dye. When the dye is injected intravenously, the eye being tapped at once after the injection is finished, the rapidity with which the dye appears in the anterior chamber varies greatly in different rabbits. It is usually from 20 to 30 minutes before fluid is obtained which would be classified as intensely stained. At this time the fluid has always coagulated spontaneously in our experience. Occasionally we have had animals in which at the end of 5 minutes the anterior chamber fluid was intensely stained. On withdrawing this at once we have several times obtained an intensely colored fluid which did not coagulate. These facts suffice to show that the appearance of the dye is independent of the appearance of at least some of the factors determining coagulation.

The distribution of the dye when it comes into the anterior chamber is interesting in that it throws some light on the rapidity of movement of the aqueous humor. In those instances where the dye appears in concentration on the iris surface before it comes through the pupil it diffuses very slowly from the point where it makes its appearance, and this diffusion is apt to be more or less even in all directions. When the dye comes through the pupil it is apt to come over the lower pupilary margin in a concentrated stream which sinks slowly to the bottom of the chamber and from there, spreads in the course of half an hour or more by diffusion until the aqueous is evenly colored.

The conditions can easily be duplicated outside the body. If one takes a thin collodion sac about an inch in diameter, fills it with a 1 per cent solution of trypan-red, and gently lowers it into a beaker of water the dye will diffuse out of the sac into the water with moderate rapidity. The dye comes out of the sac over the entire submerged surface. It then apparently creeps along the surface of the sac to the lower end of it and falls from there in a narrow stream of concentrated dye to the bottom of the beaker. It spreads along the bottom to make a concentrated red layer in this region. Then in the course of an hour or more it diffuses throughout the water. If the beaker is disturbed, of course the secondary spread is hastened.

The conditions in the anterior chamber of the eye are analogous to this, and it is hard to reconcile the observations with the view that there are any very active movements in the aqueous humor. There seems to be a tendency for matter coming into the anterior chamber through the pupil, to come in at the lower margin and to sink to the bottom of the chamber.

There is also a tendency for the dyes that we have used, at least, to leave the chamber in a definite region. If 0.5 cc. of a 1 per cent solution of trypan-red or trypan-blue is taken in a suitable syringe and the aqueous humor from a normal eye is also drawn into the same syringe and if then the mixed fluid and dye solution is immediately reinjected, care being taken to restore approximately the original tension, the absorption of dye can be watched very well. Within a few minutes vessels in the sclerotic will be seen to be injected with the dye. The vessels which are first injected and the only ones which usually show a pronounced injection are on the upper surface of the eyeball to each side of the midline. The injection of vessels in this region persists until the dye is completely absorbed.

The indication in these experiments is, then, that there is very little movement in the aqueous humor. The points of secretion and absorption are such as to determine fairly definite lines of diffusion for colloidal matter in the fluid, which in a general way are from behind the iris, through the pupil to the lower portion of the chamber, and then upward to leave at the superior portions of the angle.

Observations on the Cornea.

In a preceding paragraph we said that a number of days after an intravenous injection of trypan-red the cornea becomes distinctly colored. It is the generally accepted view that substances which reach the cornea do so by diffusion from the corneoscleral margin. The way in which the cornea becomes stained is in accord with this. If an animal is killed 2 or 3 days after the dye is injected, the cornea will, on examination, be found stained at its circumference, the colored area at this time reaching about one-third of the way to the center.

In connection with the study of experimental tuberculosis previously referred to, we observed that the reactions of the cornea were not uniform throughout. If, for example, a central inoculation is made, the first formation of blood vessels at the corneoscleral margin will be above, at approximately the midline. Next, vessels will form on the midline below, and, lastly, on the sides. We assumed that this was because substances diffused out of the cornea by preference toward the upper portion and hence stimulated the tissue reactions first at that point. That the diffusion is along these lines we have now found can be shown to be the case with the dyes we have used in this work. If the cornea is infiltrated in a spot 2 to 3 mm. in diameter at its center, the dye does not diffuse toward the lower corneoscleral margin in any appreciable degree. The diffusion is chiefly toward the upper margin, spreading out more or less in the shape of a fan in this direction. To get a diffusion chiefly toward the sides or lower margin it is necessary to place the infiltration quite close to the corneoscleral junction in those directions.

SUMMARY.

In as far as the observations reported have a bearing on the movements of fluid within the eye, they are, for the most part, in accord with views at present generally accepted. On the other hand, we know of no other way in which it may be so readily demonstrated that simple and even temporary local circulatory changes may profoundly alter the distribution of substances from the circulating

blood to the extravascular fluids and tissues. In the light of these observations, it would seem that such changes might easily account for marked idiosyncrasies in the action of poisonous drugs, and as well probably for other factors in drug action.

THE APPEARANCE OF THE PRESSOR SUBSTANCE IN THE FETAL HYPOPHYSIS.

By DEAN LEWIS, M.D.

(*From the Hull Laboratory of Anatomy, University of Chicago, Chicago.*)

Plates 96 and 97.

(Received for publication, February 21, 1916.)

It has been demonstrated (Lewis, Miller, and Matthews[1]) that the pressor substance of the posterior lobe of the hypophysis is secreted by the pars intermedia, a derivative of the pharyngeal pouch. The present study was undertaken with the hope of determining at what period in fetal life the pressor substance appears and of correlating the cytological changes in the pars intermedia with the establishment of secretory function, using the appearance of the pressor substance as an index. The cytological studies are now in progress.

I have used extracts from the entire gland. The hypophyses of pigs just before birth are large enough to permit of separation of the two lobes, but in the earlier stages this is impossible. In order to secure uniform material for injection the extracts were made from the entire gland in all instances. The glands were obtained fresh and extracted in absolute alcohol to remove the depressor substances. After extraction was completed, the alcohol was filtered off and the residue dried in a desiccator. The dried residue was kept in small bottles until desired for use, when a salt solution extract was made for intravenous injection.

Fetal pigs just before birth measure from 275 to 285 mm.; the measurement being taken from the crown of the head to the tip of the coccyx. Fig. 1 indicates the effect upon blood pressure of an intravenous injection of 0.1294 gm. of dried hypophyses removed from pigs just before birth, extracted in 5 cc. of normal salt solution.

[1] Lewis, D., Miller J. L., and Matthews, S. A., The Effects on Blood-Pressure of Intravenous Injections of Extracts of the Various Anatomical Components of the Hypophysis, *Arch. Int. Med.*, 1911, vii, 785.

This extract must be carefully filtered before injection, for much of the dried glands is insoluble. Intravenous injection of 5 cc. of this extract caused a prompt and marked rise in blood pressure which was followed by a fall and a second rise to almost the same level as the first, accompanied by marked slowing of the pulse and an increase in the length of the pulse wave. This type of tracing is often obtained after intravenous injections of extracts made from adult glands, but it is not the most common type of tracing obtained after such injections. It has been suggested that the fall following the primary rise, which is of short duration, is probably due to coronary artery contraction. This type of tracing seems to occur more frequently when extracts of the entire posterior lobe are used than when those of the pars intermedia alone are employed. It has been suggested that the variations from the common type which will be noted later may be due to the action of substances extracted from the nervous part of the posterior lobe.

Intravenous injection of 0.05 gm. of dried hypophyses from fetal pigs measuring 175 mm. extracted in 5 cc. of normal salt solution gave a decided pressor effect such as is usually obtained by the intravenous injection of extracts made from adult glands. The pressor reaction followed the injection rapidly and was long continued, being unassociated with any marked change in the tracing (Fig. 2).

Intravenous injections of saline extracts of hypophyses from fetal pigs measuring 125 mm. gave different results. In some cases a slight pressor effect following a primary fall was noted, while in other instances no change in the tracing was noted. Fig. 3 is a tracing obtained after intravenous injection of an extract of 0.08 gm. of dried hypophyses in 5 cc. of normal salt solution. The injection of this extract caused a fall in pressure which was followed by a rise slightly above the level existing before the injection was made. This reaction was of short duration, the pressure soon sinking to the level which existed before the injection was made. Another tracing was made after the injection of an extract of 0.06 gm. of dried hypophyses from pigs measuring 125 mm. extracted in 3 cc. of salt solution. The amount of extract injected was therefore practically the same as in the preceding experiment. The injection of this extract caused a fall in pressure followed by a rapid return to the

level existing before the injection was made (Fig. 4). An injection of the residue left after filtration of the preceding solution extracted in 5 cc. of normal salt solution caused another fall with a rapid return to the level existing before the injection. The fall in pressure observed in the last experiments occurs frequently after injections of glands, even after attempts to remove completely the depressor substance which exists in all parts of the gland have been made by thorough extraction with absolute alcohol.

Fig. 3 indicates that in some instances a slight but unmistakable pressor effect of short duration may follow the intravenous injection of extracts made from the hypophyses of pigs measuring 125 mm. This reaction does not follow the injection of all extracts made from the glands of pigs of this measurement. It is possible that in attempts to obtain fresh material a few pigs measuring slightly more than 125 mm. have been occasionally used, but if this is the case, they have exceeded this measurement only by a few mm.

I believe that I am justified in stating that the active principle of the pars intermedia, using the pressor substance as an index, begins to appear in fetal pigs measuring 125 mm. or slightly more.

It is somewhat difficult to determine the age of fetal pigs as estimated by measurements. Koch says that great difficulty is experienced in finding any statement concerning the age of pig fetuses. The statements of different authors do not always agree, but the two which come closest to an agreement are those of Bradley and Coe. Bradley compared the length of the embryos with the time of coition. Coe estimated the age from the rate of development of other mammals. While considerable uncertainty is attached to these figures, it may be assumed that the 50 mm. pig fetus is about 40 days old from conception; the 100 mm. fetus is 55 to 62 days old; and the 200 mm. pig is 88 to 90 days old from conception.

As determined by the pressor reaction the secretion of the pars intermedia seems to be established in pigs measuring 125 mm. The reaction is not obtained after the injection of all extracts of glands removed at this period and even when present is not marked. There are, however, often definite indications of a pressor effect as indicated in Fig. 3 As far as can be estimated, a pig measuring 125 mm. is about 9½ or 10 weeks old from conception. During the period rep-

resented by the differences between 125 and 175 mm., the secretion of the pars intermedia becomes as active as that of the adult gland.

McCord[2] has recently attempted to determine the time of appearance of the active principle of the pars intermedia. Its presence was determined by the oxytocic activity of extracts of glands by means of the method of Dale and Laidlaw,[3] using histamine as a standard. The tests for pituitrin were begun with embryos at or near full term. These tests and others as far back as 9 weeks were quantitative tests. Pituitrin was found in the extracts of glands as early as 9 weeks, and the quantity for unit of weight was larger than in the adult. In the 7th and 8th weeks of fetal life the pituitary could no longer be recognized, although the sella turcica was plainly visible. The experiments, therefore, could not be made earlier than the 9th week of fetal life.

McCord worked with bovine fetuses and it is difficult to determine the relative ages of calf and pig fetuses by the lengths. McCord found that the first indication of an active pars intermedia occurs in a bovine fetus measuring 165 mm., while evidences of an active pars intermedia are found in a pig fetus measuring 125 mm.

Fenger[4] has shown that both the thyroid and suprarenal glands of the beef, hog, and sheep contain their active principles not only at birth, but also in the fetus. He believes that both the thyroid and suprarenals of the fetus take a distinct and active part in its growth.

SUMMARY.

The pressor substance of the hypophysis is so marked in the pig fetus measuring 175 mm. that it seems probable that a fetus of this length is independent of the secretion of the mother's hypophysis.

[2] McCord, C. P., The Occurrence of Pituitrin and Epinephrin in Fetal Pituitary and Suprarenal Glands, *Jour. Biol. Chem.*, 1915, xxiii, 435.

[3] Dale, H. H., and Laidlaw, P. P., A Method of Standardizing Pituitary (Infundibular) Extracts, *Jour. Pharm. and Exper. Therap.*, 1912–13, iv, 75.

[4] Fenger, F., On the Presence of Active Principles in the Thyroid and Suprarenal Glands Before and After Birth. Second Paper, *Jour. Biol. Chem.*, 1912, xii, 55.

EXPLANATION OF PLATES.

Plate 96.

Fig. 1. Effect upon blood pressure of the intravenous injection of 0.1249 gm. of dried hypophyses removed from pigs just before birth, dissolved in 5 cc. of normal salt solution. This type of tracing is often produced by injection of extracts of the adult gland, but is not the most common form.

Fig. 2. Effect upon blood pressure of the intravenous injection of 0.05 gm. of dried hypophyses removed from pigs measuring 175 mm., dissolved in 5 cc. of normal salt solution. A prompt and long continued pressor effect is noted.

Plate 97.

Fig. 3. Effect upon blood pressure of the intravenous injection of 0.08 gm. of dried hypophyses removed from pigs measuring 125 mm., dissolved in 5 cc. of normal salt solution. The fall in pressure is followed by a distinct but temporary rise.

Fig. 4. Effect upon blood pressure of the intravenous injection of 0.06 gm. of dried hypophyses removed from pigs measuring 125 mm., dissolved in 3 cc. of normal salt solution. A fall in pressure is noted with a rise to the level existing before the injection.

CHEMICAL VERSUS SERUM TREATMENT OF EPIDEMIC MENINGITIS.

By SIMON FLEXNER, M.D., and HAROLD L. AMOSS, M.D.
(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 20, 1916.)

INTRODUCTION.

The reappearance of epidemic meningitis among the belligerent armies in Europe has led to numerous reports on the treatment of the disease. For the most part, the antimeningococcic serum has been employed, but in one instance at least the attempt has been made to improve upon the serum treatment by substituting for it chemical treatment. This attempt to employ directly antiseptic drugs in the treatment of epidemic meningitis is a revival of an old notion.

The circumstances are such as to make the chemical treatment favorable from a theoretical standpoint. The infection tends to be local, that is, within the cerebrospinal membranes, and not general or throughout the body. The membranes are directly accessible from without, so that the chemical substance can be brought into immediate relation with the seat of infection. Moreover, the meningococcus as cultivated outside the body is not a highly resistant microorganism, but is readily injured by chemical action, and is indeed quickly injured by the waste products of its own growth. What, therefore, is apparently simpler or more promising than to control epidemic meningitis by the direct application by means of lumbar puncture of bactericidal chemicals to the infected and inflamed cerebrospinal membranes?

There is, moreover, still another theoretical reason for resorting to the chemical treatment of epidemic meningitis. The meningococcus is now known to be a microorganism which occurs in at least two varieties or types, distinguished as meningococcus and parameningococcus.[1] Their main differences are immunological, so that they

[1] Amoss, H. L., and Wollstein, M., *Jour. Exper. Med.*, 1916, xxiii, 403.

are subject to therapeutic influences through antisera only when correspondence exists between the infecting type of microorganism and the particular specific antibodies present in a serum. Presumably, chemical agents being less specific would be less restricted in their effects, and might be expected to act quite irrespective of the type of meningococcus causing the infection.

Wolff[2] has recently recommended the employment of protargol in the treatment of epidemic meningitis, basing the recommendation of its use on eight cases, of which five recovered. The point of departure in the selection and administration of protargol was the successful employment of so called colloidal silver preparations in the treatment of gonorrhea, and the close relationships as regards conditions of culture, survival, and reaction to antiseptics subsisting between gonococci and meningococci. It is conceded by Wolff that because of the highly variable clinical course of epidemic meningitis, the number of cases which he reports is too small to permit of the drawing of any conclusions regarding the therapeutic efficiency of the chemical. He believes, however, that it is at least innocuous and can, therefore, be administered with the assurance that if it is not curative, it will do no harm.

The first method of direct chemical treatment of epidemic meningitis to receive considerable attention was that in which lysol was employed. The method was introduced in Lisbon during the epidemic which prevailed there in the first years of the present century, and was briefly reported on by Seager[3] in 1902. Following Seager's report, lysol was tested in the course of the epidemic from which Greater New York was suffering at the time.[4] This epidemic continued for several years and led to the working out of the serum treatment, but at that early date no specific means of combating the disease was known.

It is remarkable and instructive to consider that in these instances, namely, the one relating to lysol and the other to protargol, what appeared to be preliminary success was achieved by the chemical treatment. Wider experience, however, led to the abandonment of

[2] Wolff, G., *Deutsch. med. Wchnschr.*, 1915, xli, 1486.

[3] Seager, H. W., *Lancet*, 1902, ii, 1188.

[4] Manges, M., *Med. News*, 1904, lxxxiv, 913.

the lysol treatment. In the meantime, knowledge has been gained and methods perfected through which the value of a proposed form of treatment of epidemic meningitis can be determined experimentally in animals, with a degree of accuracy exceeding that based on clinical and limited statistical observations on human beings.

It happens that epidemic meningitis in man is a highly variable disease. The severity fluctuates between clinical conditions so slight as to justify the term abortive being applied to certain cases, and at other times so severe (fulminant) as to cause death swiftly, and sometimes with the first appearance of symptoms. Hence the mortality percentage of the disease varies in different epidemics and places. To determine approximately what the effects of varying modes of treatment are it is necessary to compare coincidently treated and untreated cases of sufficient number. A definite result is therefore obtained only after some time, and by comparing figures obtained in different localities and over a comparatively long period of time. So long as the figures based on small local observations alone are available, no final deduction can be made.

The employment of animals for the test is free of the uncertainty of the statistical method. The dosage of the culture of meningococcus can be adjusted to produce the result desired. Then the effect and the manner of the therapeutic action can be accurately ascertained. Tests of this kind have been made with protargol and for comparison with lysol and with serum, with results unfavorable to the chemical treatment.

EXPERIMENTAL.

The experimental studies on epidemic meningitis carried out during the past 10 years have shown two kinds of animals to be suitable for determining the pathogenic action of meningococci and the effects of therapeutic agents in meningococcic infection. The animals are monkeys[5] and young guinea pigs.[6] While the age of the monkeys seems not to matter, older guinea pigs are highly resistant to meningococcic infection. The cultures of meningococci are injected into

[5] Flexner, S., *Jour. Exper. Med.*, 1907, ix, 142.

[6] Flexner, S., *Jour. Exper. Med.*, 1907, ix, 168.

the peritoneal cavity in the guinea pigs and into the subarachnoid space in the monkeys, in the latter by means of lumbar puncture. The injected meningococci set up local inflammations in the exudate attending which the fate of the microorganism can be followed precisely, as in human cases of epidemic meningitis in which the cerebrospinal fluid is removed from time to time by lumbar puncture.

The changes occurring in the meninges or the peritoneum vary with the activity and the dose of the culture. Non-virulent cultures are quickly phagocyted, no considerable multiplication takes place, and the inoculated animals show few or no symptoms and recover fully in 24 hours. Active cultures multiply, are imperfectly phagocyted, and cause death according to the virulence and dose in 6 to 8 to 12 hours in guinea pigs, and in 20 to 48 hours in monkeys. At autopsy living meningococci are present in the peritoneal or meningeal exudate and in the blood. Film preparations show meningococci free and phagocyted also in the peritoneal fluid and more numerously in the omentum of the guinea pig, and both free and phagocyted in the exudate in the pia-arachnoid membranes of the monkey.

In carrying out the experiments a virulent strain of the meningococcus (Isadore) was employed. Tests made to determine the optimum period of growth at which to employ the culture indicated it to be after about 18 hours' incubation at 37°C. Sheep serum dextrose agar was used as a medium. Growths 24 hours old infected far more irregularly than those 18 hours old. The reason for the disparity is found in the spontaneous degeneration of the culture, which probably has already begun at 18 hours, and can be detected by microscopic examination at 22 hours. In the guinea pigs it was found in this instance, as already described,[1] that consistent results are best attained by employing one minimal lethal dose of the culture. In this way, lack of protective power is made to assert itself readily, while the existence of protective power is easily demonstrated and can be further investigated. In monkeys the infecting dose is easily determined, although the fatal dose is established less readily. While these animals were used sparingly, the protocol in each instance indicates the outcome of the therapeutic test compared with the control. Besides the result based on death or survival, the changes

taking place in the cerebrospinal fluid as followed by lumbar puncture, and the clinical course of the experimental disease, to a less definite degree, are also valuable indications of the action of the therapeutic agent.

Tests with Guinea Pigs.

The guinea pigs ranged in weight from 90 to 110 gm. The injections were intraperitoneal. The protargol was employed in 0.2 per cent strength, prepared by suspending the dried powder in sterile distilled water. The cultures of meningococci on sheep serum agar after 18 hours' incubation were suspended in sterile salt solution, and injected either immediately or after such intervals as are indicated in the separate experiments. The dose was one m.l.d.

TABLE I.

Experiment 1. Toxicity of Protargol.

Weight of guinea pig.	Quantity of suspension injected.	Result.
gm.		
90	0.3 cc. + 0.7 cc. water.	Survived.
90	0.5 " + 0.5 " "	"
90	1.0 " + 1.0 " "	"
90	2.0 " + 2.0 " "	Died in 38 hrs.

The tests shown in Table I may be taken to indicate that the toxic dose of protargol for young guinea pigs is well above the dose employed for therapeutic purposes.

Experiment 2. Therapeutic Tests of Protargol Intraperitoneally in the Guinea Pig.—One m.l.d. of living culture in a total volume of 1 cc. was injected intraperitoneally into young guinea pigs. The amount of 0.2 per cent protargol suspension given was made up to 1 cc. with sterile water and injected at the time indicated in Table II.

TABLE II.

Weight of guinea pig.	Dose of protargol suspension.	Time of administration.	Result.
gm.	*cc.*		
110	0.5	Immediately.	Death in 12 hrs.
110	0.5	"	" " 21 "
95	0.5	"	" " 15 "
100	0.5	"	" " 10 "
99	0.1	"	" " 8 "
82	0.1	"	" " 19 "
82	0.1	"	" " 11 "
100	0.5	After 15 min.	" " 12 "
110	0.7	" 15 "	" " 8 "
100	0.5	" 1 hr.	" " 10 "
75	0.1	" 1 "	" " 15 "
75	0.1	" 1 "	" " 18 "
95	0.25	Mixed with culture, injected after 1 hr.'s contact.	" " 25 "
110	1.00	" " "	" " 14 "

This experiment is conclusive in demonstrating that protargol in 0.2 per cent suspension, the strength employed by Wolff,[2] is incapable of preventing in guinea pigs the lethal effect of a single minimal fatal dose of the meningococcus, whether administered combined with the culture immediately after the mixture, or whether the culture and protargol are injected separately 15 minutes apart. The period of survival of the treated animals may be about the same as or somewhat greater than the controls. Living meningococci are always found in the peritoneal cavity and in the heart's blood. In this connection it is interesting to find that when the culture and protargol suspension which have been in contact for 1 hour are transplanted to fresh sheep serum agar, no growth is obtained; while, however, cultures from the peritoneal cavity of the dead animals which were inoculated with the mixture are positive, thus indicating that the protargol merely inhibits the growth of the meningococci, but may not destroy them in 1 hour's time. This observation raises the question whether the failure of the protargol is due merely to its imperfect bactericidal power, or whether it depends upon lack of antitoxic power at the same time. The next experiment bears on this consideration.

Experiment 3. Therapeutic Test of Protargol with Autolysate.—An autolysate[7] of the meningococcus was prepared in the usual way by suspending the culture in salt solution, adding toluene, incubating, allowing the toluene to evaporate, and centrifugalizing. The clear supernatant fluid was employed. The autolysate and protargol suspensions were mixed and immediately injected, as shown in Table III.

TABLE III.

Weight of guinea pig.	Dose of autolysate.	Dose of suspension of 0.2% protargol.	Result.
gm.			
90	0.25 cc. in 1 cc. salt solution.	None.	Died in 7 hrs.
100	0.25 " " 1 " " "	0.5 cc.	" during night.
110	0.25 " " 1 " " "	1.0 "	" " "

This experiment indicates that protargol does not exhibit pronounced antitoxic properties, although a delay was noted in the fatal issue in the animals in which it was given.

Mechanism of the Action of Protargol and of Antiserum.

In carrying out the above tests which were made on several different occasions, control observations with antimeningococcic horse serum were also made. The quantity of serum administered was 0.5 cc., and in every one of the six instances in which the antiserum was given the animal survived. The antiserum was also injected in association with the protargol, without, however, preventing a fatal issue. On the other hand, the antiserum influences directly the mechanism of action of protargol, although unable apparently to overcome wholly some injurious effect which it exerts. The next experiment, therefore, was devised to bring out definitely the mechanism of action of the protargol in comparison with the manner of action of the antiserum.

Experiment 4. Mechanism of the Action of Protargol.—Four guinea pigs, ranging in weight from 90 to 110 gm., were employed. Besides the inoculation of meningococci, one of them received immediately 0.5 cc. of a suspension of 0.2 per cent protargol alone; the second received 0.5 cc. of antiserum alone; and

[7] Flexner, S., *Jour. Exper. Med.*, 1907, ix, 105.

the third 0.5 cc. of a suspension of protargol and 0.5 cc. of antiserum in immediate succession. The fourth animal received only the culture. All were chloroformed at the expiration of 6 hours, at which time the control animal was prostrate and the others were definitely ill.

The autopsies were made at once and the distribution of meningococci in the abdominal cavity studied by means of cultures and film preparations, and in the blood of the heart by cultures alone.

Guinea Pig 1. Protargol Alone.—Two drops of the blood of the heart were laked in 0.5 cc. of sterile water and plated in sheep serum agar. The colonies numbered about 2,000. Two drops of the slightly turbid peritoneal fluid were similarly plated. The colonies numbered about 2,000. The film preparations from the fluid in the abdominal cavity showed numerous scattered meningococci and a notable absence of polynuclear cells, and the omentum showed scattered free meningococci and polynuclear leukocytes, of which a very small number contained diplococci, the leukocytes themselves staining feebly and appearing degenerated.

Guinea Pig 2. Antiserum Alone.—Two drops of the heart's blood were treated as in No. 1. No colonies. Two drops of the somewhat turbid peritoneal fluid gave about 2,500 colonies. Film preparations of the peritoneal fluid showed numerous well preserved polynuclear leukocytes containing many meningococci, while the extracellular diplococci, small in number, were arranged in small clumps. The film preparations from the omentum exhibited many more leukocytes, most of which were filled with meningoccoci in various stages of disintegration, the leukocytes themselves appearing in normal condition. A few extracellular agglutinated meningococci were also present.

Guinea Pig 3. Protargol and Antiserum.—Two drops of the heart's blood yielded one colony, and two drops of the clear peritoneal fluid about 150 colonies. Film preparations of the peritoneal fluid showed a smaller number of diplococci than in either of the preceding animals. Polynuclear leukocytes were present, but in far smaller number than in No. 2, and many were degenerated and had rarely phagocyted the diplococci. Such meningococci as were present at all were agglutinated. The films from the omentum showed somewhat larger numbers of degenerated polynuclear cells, of which a part contained meningococci, and quite numerous free, single and clumped diplococci.

Guinea Pig 4. Control.—Two drops of the heart's blood gave about 2,000 colonies, and two drops of the clear peritoneal fluid innumerable colonies. Congestion of the peritoneal organs was more marked in this animal than in the others. Film preparations of the peritoneal fluid showed a very large number of particularly well preserved meningococci, but no polynuclear leukocytes, and those of the omentum showed a similar condition. The absence of polynuclear cells from the latter structure is particularly noteworthy.

This experiment confirms the observation made upon the guinea pigs treated with protargol which were allowed to succumb. Its

significance is clear. Protargol has some power to cause dissolution of the meningococci with which it comes in contact, but does not destroy all. A later increase occurs and invasion of the blood stream takes place. Probably the injurious action of the protargol arises from its antileukotactic properties, for it causes degeneration of the polynuclear leukocytes and prevents phagocytosis, both *in vivo* and *in vitro*.

This particular action of the protargol is rendered obvious at once on comparison with the manner in which the antiserum acts. In this instance there is a diversion of polynuclear leukocytes in large numbers to the peritoneal cavity and an acceleration of phagocytosis. Hence at a period at which many living meningococci are still present in the abdominal cavity, practically no escape is taking place into the general blood stream. The addition of the antiserum to the protargol obviates partially the antileukotactic effect of the latter, but does not completely overcome it, because of the injury inflicted by the chemical on the leukocytes brought out by the antiserum.

Protargol is, therefore, bactericidal for the meningococcus, as for the gonococcus, through direct chemical action, but it is not curative in meningococcic infection in the guinea pig, because being confined within the abdominal cavity it exerts a passive chemotactic and antiphagocytic influence on the polynuclear cells which are themselves injured and destroyed through its action. Thus the imperfect bactericidal effect is inadequate to control infection, while the antileukotactic action is itself harmful and promoting of infection. Moreover, the masses of protargol are themselves taken up by leukocytes, to be transported doubtless to organs in which the substance may remain indefinitely.

Tests with Monkeys.

The inoculation of virulent meningococci into the subarachnoid space in monkeys causes an acute inflammation which extends throughout the meninges and into the cerebral ventricles. Depending upon the pathogenic power and the dose, the animal which develops symptoms within a few hours may die within 24 hours or may recover. According as one or the other result is obtained, the meningococci multiply and are imperfectly phagocyted, or they increase little

and are perfectly phagocyted. The cerebrospinal fluid at first remains clear, then becomes turbid with emigrated polynuclear leukocytes; finally it may again become clear. A blood invasion often follows within a few hours of the inoculation, but is rarely present at the end, even in fatal cases.

Experiment 5. Control.—A *Macacus rhesus* was injected at 11 a.m. intraspinally with 2 cc. of a salt solution suspension, representing 1½ agar slant cultures of meningococcus, the strain being the same as that used in the experiments on the guinea pigs. 5.00 p.m., animal sick. Lumbar puncture yielded a fluid already turbid, giving positive culture and showing on films many meningococci, few leukocytes, and little phagocytosis. Blood taken from arm vein gave positive cultures. 9.00 a.m. the next day, prostrate. Lumbar puncture yielded highly turbid fluid, giving positive cultures and films in which leukocytes were numerous and of which many contained meningococci; free diplococci were also present. Death at 3.30 p.m. Survived 20½ hours.

Autopsy.—The membranes of the brain and spinal cord showed turbid fluid and congestion. The cultures and film preparations confirmed previous findings. Both intra- and extracellular meningococci were present. Cultures positive. Cultures of heart's blood negative.

Sections from the brain and spinal cord show an acute fibrinopurulent meningitis, most pronounced over the convex surface of the brain. The ventricles are involved slightly. Many diplococci are present in the exudate and are contained chiefly, if not wholly within leukocytes.

Experiment 6. Treatment with Antimeningococcic Horse Serum.—8.30 a.m., a *Macacus rhesus* received intraspinally an injection of a suspension of meningococci as in Experiment 5. 12.30 p.m., lumbar puncture yielded cloudy fluid, giving positive cultures. Films showed numerous meningococci, pus cells, and phagocytosis already present. The culture from the heart's blood was negative. 2 cc. of antiserum were injected. 4.30 p.m., animal appeared well; lumbar puncture yielded turbid fluid, giving positive culture and showing in the film marked phagocytosis and agglutination of all extracellular meningococci. 8.30 p.m., active. 28 hours after the inoculation the animal appeared well; lumbar puncture yielded a turbid fluid which gave a negative culture. The films showed many leukocytes, some containing meningococci and very few extracellular diplococci. A number of endothelial cells and lymphocytes were present. The animal remained well.

Experiment 7. Treatment with Protargol.—8.30 a m., a *Macacus rhesus* received an equal part of a suspension of the culture used in the previous experiments. 12.30 p.m., little change in condition. Lumbar puncture yielded a cloudy fluid, from which cultures were positive and films show many leukocytes and well advanced phagocytosis of meningococci; some free meningococci were also present. Injected 2 cc. of 0.2 per cent protargol suspension. 2.30 p.m., animal

lying down, drowsy, surface cold, limbs spastic. 4.00 p.m., respiration rapid, slight convulsion recurring frequently. Blood withdrawn at this time gave a positive culture. 4.30 p.m., died. Survived 8 hours.

Autopsy.—No lesions were found outside the cerebrospinal membranes. The meninges contained an excess of almost clear fluid. No marked congestion. Cultures from the meninges of the brain and spinal cord yielded luxuriant growth. Films showed a large number of meningococci, both intra- and extracellular, and a widespread degeneration and even disintegration of leukocytes. The meningococci stain sharply and deeply. The choroid plexus of the lateral ventricle and the meninges of the olfactory lobes gave results similar to those of the other membranes. Cultures from the heart's blood were positive. Moreover, cultures prepared from the nasal mucosa yielded, among other bacteria, typical meningococci, possessing identical agglutination reactions with the strain employed for inoculation.

The microscopic examination of sections from the central nervous organs shows many pus cells, with here and there red corpuscles, and no fibrin. The number of meningococci present is very large. Some degree of phagocytosis exists; but a striking phenomenon is the packing of lymph spaces with innumerable diplococci. They invade the pial spaces, infiltrate the superficial layers of the cerebral cortex, and follow the perivascular spaces for some distance into the cortex. Certain coagula of red and white corpuscles within the central ventricles also enclose diplococci.

A comparison of the results in the three monkeys inoculated with meningococci and left in one case untreated, and in the others treated either with antiserum or protargol, is unfavorable to the treatment with protargol. The untreated or control animal succumbed under conditions indicating definite if inadequate reaction against and resistance to the infection. The serum-treated animal was already ill when the antiserum was administered, the effect of which was to disperse almost immediately all the symptoms and lead to prompt recovery, coincidently with the removal by phagocytosis of the meningococci. The third monkey exhibited practically no symptoms 4 hours after the inoculation when the protargol was given, but prostration and rapid intensification of symptoms followed almost immediately. Death occurred 4 hours later. The causes of the unfavorable results are to be sought in the two or three main effects of the protargol: (*a*) on the leukocytes, which already present, were greatly injured; (*b*) on the meningococci, which doubtless were also affected through direct bactericidal action from which toxic substances were set free; and (*c*) finally on removal of all restraint to

multiplication of surviving diplococci, since the immense numbers present in the lymph spaces as well as in the exudate indicate rapid growth.

A repetition of the experiment in which protargol was used was made in Experiment 8.

Experiment 8. Treatment with Protargol.—11.15 a m., a *Macacus rhesus* received intraspinal inoculation of one agar slant culture, 18 hours old. 3.15 p.m., slightly ill; on perch. Lumbar puncture yielded cloudy fluid; culture positive. Films showed few leukocytes and many free meningococci. Injected 2 cc. of 0.2 per cent protargol suspension. 4.00 p.m., animal prostrate and spastic. 5.00 p.m., refused to rise. Died 5.00 a.m. next day. Survived 18 hours.

Autopsy.—No obvious visceral lesions outside the central nervous system. Turbid fluid in meninges, especially evident in sulci of cerebral convolutions. The general gray color of the cortex was about normal, but two symmetrical rectangular areas measuring 2 by 4 cm. were deeply congested and contained petechial hemorrhagic spots. They lay anterior and posterior to the fissure of Sylvius and extended to the midparietal sulcus comprising the ascending and inferior marginal convolutions. A few separate hemorrhages existed in the first temporal lobe, and the congestion and hemorrhages appeared to be confined to the gray matter. Cultures from the heart's blood and from several levels of the spinal cord and different surfaces of the brain were positive for the meningococcus. Film preparations showed many diplococci, few if any leukocytes, and little phagocytosis. Impression films prepared from the congested area of the brain contain scattered meningococci, few leukocytes, and almost no phagocytosis.

Sections from the brain show edema of and many leukocytes and red corpuscles within the meninges. The axudate contains many diplococci, both extra- and intracellular. In places the diplococci extend into the superficial layer of the cortex and slightly into the perivascular lymph spaces. These are far less numerous than in the 8 hour specimens. The affected cortex is congested and is the seat of many hemorrhages of varying size. The escape of blood is partly into the tissues and partly into the perivascular lymphatics. There is no obvious special relation between the hemorrhages and the diplococci.

A control for this monkey which received an identical quantity of an 18 hour culture was made ill, but recovered without treatment and following the spontaneous phagocytosis of the meningococci.

The differences in general disadvantageous to the protargol arose also in instances in which neither the untreated control nor the treated monkeys succumbed. In these instances cultures 24 hours old were employed. The symptoms set in more slowly than when 18 hour cultures were used and became less severe, abating wholly later.

But the monkeys given protargol suffered intensification of the symptoms almost immediately after the injection and recovered much more slowly than the untreated animals. In conformity with this fact it was found that the meningococci survived longer in the cerebrospinal fluid in the treated animals than in the control. A single experiment is given to bring out the distinction mentioned.

Experiment 9. Control.—8.50 a.m., a *Macacus rhesus* received an intraspinal inoculation of $1\frac{3}{4}$ agar slant cultures of meningococci. 2.50 p.m., slightly ill. Turbid lumbar puncture fluid gave growth. The films showed numerous leukocytes, few meningococci, and little phagocytosis. Blood cultures were negative. The symptoms progressed slowly and never became severe. 24 hours after inoculation, the animal was still ill, but improving. Lumbar puncture yielded turbid fluid, which gave negative cultures. Film preparation of the fluid showed numerous leukocytes and few meningococci all within phagocytes. Within another 24 hours the animal had recovered.

Experiment 10. Treated.—8.30 a.m., a *Macacus rhesus* received intraspinally $1\frac{1}{2}$ agar slant cultures. 12.30 p.m., ill. The turbid lumbar puncture fluid gave positive cultures. The film showed many leukocytes, few meningococci, and slight phagocytosis. Injected 1 cc. of 0.2 per cent protargol suspension. 1.30 p.m., very ill. 5.00 p.m., condition unchanged; blood culture positive. Turbid lumbar puncture fluid gave positive cultures. Film showed many leukocytes, of which some contained meningococci; many extracellular meningococci also. 10.00 p.m., animal lying on bottom of cage; aroused with difficulty. 24 hours after inoculation, improving. Turbid lumbar puncture of fluid still showed intra- and extracellular diplococci. No growth was obtained in culture. At the expiration of another 24 hours, the animal had still further improved, but several days elapsed before recovery was complete.

Effects of Lysol.

The fact has already been mentioned that lysol has been employed in the treatment of epidemic meningitis with at first, as was supposed, beneficial results. Although a wider employment led very soon to its abandonment, yet the outcome of the studies of protargol suggested determining also the manner in which lysol acts. For this purpose small guinea pigs were employed. Two sets of tests were made: one in which the inoculated animals were allowed to succumb, the other in which they were chloroformed at the expiration of 6 hours, in order to ascertain just what changes were taking place in the peritoneal cavity. The lysol was employed in

1 per cent solution, which was the strength used in human cases of meningitis. The injection was made immediately following the injection of the culture, both being given into the peritoneal cavity (Table IV).

TABLE IV.

Experiment 11. Therapeutic Test of Lysol.

Weight of guinea pig.	Quantity of culture inoculated.	Treatment.	Result.
gm.			
100	1 m.l.d.	None (control).	Died in $10\frac{3}{4}$ hrs.
100	1 "	1 cc. lysol (control).	Survived.
100	1 "	0.5 cc. lysol.	Died in 7 hrs.
100	1 "	0.5 cc. lysol + 0.5 cc. antiserum.	" " 32 "
100	1 "	0.25 cc. lysol + 0.5 cc. antiserum.	" " 20 "
100	$\frac{1}{2}$ "	0.5 cc. lysol.	" " 12 "

Guinea Pig 1. Control.—Peritoneal fluid clear. 0.05 cc. plated gave innumerable colonies of meningococci, as did the same quantity of heart's blood. Films of peritoneal fluid showed enormous numbers of meningococci, but no cells. Films from the omentum showed extremely few leukocytes containing meningococci.

Guinea Pig 2.—Considerable amount of clear peritoneal fluid. 0.05 cc. plated gave innumerable colonies. Same amount from heart's blood gave 250 colonies. Films of the peritoneal fluid showed large numbers of diplococci, well preserved but agglutinated into small clumps. No leukocytes present. Films of the omentum showed a considerable number of leukocytes, many containing diplococci and many extracellular diplococci agglutinated.

Guinea Pig 3.—Very small amount of turbid peritoneal fluid. 0.05 cc. plated gave innumerable colonies. Same quantity of heart's blood gave 200 colonies of meningococci. Films of peritoneal fluid showed numerous leukocytes filled with diplococci, and large numbers of diplococci arranged in small clumps remaining extracellular.

Guinea Pig 4.—Small amount of turbid peritoneal fluid. 0.05 cc. plated gave 1,000 colonies. Same quantity of heart's blood gave no colonies. Films of peritoneal fluid showed numerous leukocytes, some staining badly, many containing diplococci, and a considerable number of small clumps of extracellular diplococci, some partly disintegrated. Films of the omentum showed more phagocytosis and a smaller number of extracellular diplococci.

Guinea Pig 5.—A considerable amount of turbid peritoneal fluid. 0.05 cc. plated gave innumerable colonies, as did the heart's blood. Films of the peri-

toneal fluid showed many leukocytes and a small amount of phagocytosis, besides extracellular clumps of diplococci. Films of the omentum showed more phagocytosis than the peritoneal fluid and a larger number of leukocytes, besides extracellular diplococci.

Guinea Pig 6.—A considerable amount of turbid peritoneal fluid. 0.05 cc. plated gave innumerable colonies, as did the heart's blood. Films of the peritoneal fluid showed numerous leukocytes, a small amount of phagocytosis, and numerous clumped extracellular diplococci. Films of the omentum showed numerous leukocytes, slight phagocytosis, and extracellular diplococci occurring in clumps.

Mechanism of the Action of Lysol.

The mode of the action of lysol was studied by inoculating three young guinea pigs with one minimal lethal dose of culture and treating them immediately with 1 per cent lysol or 1 per cent lysol plus antiserum, as indicated in Table V.

TABLE V.

Experiment 12.

Weight of guinea pig.	Quantity of culture inoculated.	Treatment immediately after inoculation.	Condition after 6 hrs.
gm.			
100	1 m.l.d.	0.5 cc. lysol + 0.5 cc. water.	Dead.
100	1 "	0.25 " " + 0.75 " "	Very sick.
100	1 "	0.25 " " + 0.25 " " + 0.5 cc. antiserum.	Prostrate.

The two guinea pigs which still survived were chloroformed and autopsies performed at once.

Guinea Pig 1.—Peritoneal fluid clear. 0.05 cc. plated gave innumerable colonies of meningococci. Same quantity of heart's blood gave 100 colonies. Films of the peritoneal fluid showed many meningococci and few leukocytes, there being almost no phagocytosis, while the leukocytes stained feebly. The meningococci were chiefly aggregated into clumps of 10 or 20 individuals. They stained as a rule diffusely and not sharply. The omentum showed many more leukocytes, almost all containing meningococci singly and in small clumps. The phagocytes stained feebly.

Guinea Pig 2.—Peritoneal fluid clear. 0.05 cc. gave innumerable colonies of meningococci. Same quantity of heart's blood gave about 100 colonies. Films of peritoneal fluid and omentum showed a condition similar to No. 1.

Guinea Pig 3.—Peritoneal fluid turbid, gave 150 colonies of meningococci and the same quantity of heart's blood gave three colonies. Films from the peritoneal cavity showed no diplococci whatever, either free or in leukocytes, of which a good number in excellent preservation were present. Films from the omentum showed large numbers of well preserved leukocytes, of which a few contained meningococci, either sharply stained or disintegrating; extracellular diplococci were practically absent.

It may in brief be stated that the experiments with guinea pigs indicate that lysol is antileukotactic to an even greater degree than is protargol. Moreover, the antiserum is capable in this case of overcoming to even a further extent the negative chemotactic and antiphagocytic action of the chemical. Obviously the combined toxic power of the chemical and the disintegrating effect upon the meningococci, notwithstanding the action of the serum, bring about a fatal issue.

DISCUSSION.

The results of the tests with protargol and with lysol are consistent. The chemicals have shown themselves not to be curative, but rather to be injurious in experimental meningococcic infection in guinea pigs and monkeys. Far from exhibiting power to convert a fatal into a non-fatal issue, they have shown rather a reverse tendency. Even when, as in certain tests on monkeys, recovery occurred after employing protargol, the symptoms of the treated animals were intensified as compared with the untreated control animals.

The explanation of the unfavorable effect of the chemicals is supplied by the study of the processes occurring in the peritoneum of guinea pigs and the subarachnoid space of monkeys. The key to recovery from infection with the meningococcus is furnished by the phenomenon of phagocytosis. Whatever means promote phagocytosis under conditions in which the leukocytes remain potent, facilitate recovery; whatever means lead to the reverse effect interfere with recovery. The chemicals have been shown to be antileukotactic, antiphagocytic, and indeed to be cell poisons of considerable power. Hence when they are brought into relation with the seat of infection, they prevent emigration of leukocytes on an adequate scale, and they reduce phagocytosis by such leukocytes as have entered the serous cavities.

Moreover, they also injure and bring about degeneration of the leukocytes themselves.

The resultant of this set of injurious activities is to open the way for a free invasion of the blood by the meningococci and for almost unrestrained multiplication in the serous cavities.

The chemicals do exert a direct bactericidal action upon such meningococci as come under their influence under conditions of suitable concentration, but not all the meningococci are thus destroyed. Those which survive multiply almost without hindrance; while the absence of detoxicating power on the part of the chemical permits the disintegration products of the destroyed meningococci to exert their poisonous effect upon the animal organism.

The manner of action of the chemicals is precisely the reverse of the antiserum. The latter acts leukotactically and brings into the infected serous cavities a far larger number of leukocytes than would otherwise appear there in the same period of time. Moreover, by preparing the meningococci for phagocytosis by supplying opsonin and by facilitating that process by agglutinating the diplococci, the serum greatly promotes the englobing of the microorganisms. Finally, through the possession of antitoxic power it neutralizes whatever endotoxin may be liberated by the disintegrating diplococci.

It is, therefore, not remarkable to find, as the experiments have shown, that the antiserum is capable of overcoming part of the defects of the chemicals. Because of its leukotactic and phagocytic properties, the antiserum removes some of the damage which the chemicals produce on account of their antagonism to those essential phenomena. But combinations of the chemical and the antiserum have proven less effective in combating experimental meningococcic infection than the antiserum alone, from which the deduction may be made that whatever benefit may attach to effective direct bactericidal action by the chemicals, irrespective of the type of meningococcus causing the infection, is lost because of the dangers arising from their antiphagocytic effects and lack of detoxicating properties.

Doubtless the introduction of the chemicals into the subarachnoid space in man has been made without the uniformly serious consequences observed in our experiments on animals. The reason for this

discrepancy is perhaps obvious. The relative dosage, considering comparative size and weight, was far less in man than in the animals. Hence it may be assumed that the injurious effects, if any were produced, were masked. To assume, on the other hand, that because no evidence of untoward action was detected in the treatment with protargol, and five out of eight cases of epidemic meningitis treated with that chemical recovered, the drug has curative properties, is to disregard previous experience with simple lumbar puncture, with lysol, and with other methods of treatment which for a time and because of the variable severity of cases of epidemic meningitis seemed to offer encouragement. It is not probable that such active protoplasmic poisons as protargol and lysol can be employed with impunity for direct introduction into the closed cavity of serous membranes, the seat of the meningococcic infection.

SUMMARY.

Claims of efficiency have been made at two widely separated periods for the chemical treatment of epidemic meningitis, in the first instance for lysol and in the second for protargol. The use of lysol was long since abandoned; the recommendation for protargol is based on a single series of cases, small in number. Because of the variable severity of epidemics of meningitis, small reliance can be placed on results of treatment limited in extent to small numbers of cases and to one locality. A more uniform and accurate measure of the value of a method of treatment is provided by animals infected experimentally with pathogenic cultures of meningococci.

Young guinea pigs respond in a definite manner to intraperitoneal inoculation of virulent meningococci. Neither protargol nor lysol proved to have any curative action on the experimental infection thus produced in these animals.

Monkeys respond in a characteristic manner to the inoculation of virulent cultures into the subarachnoid space. Protargol displayed no curative action on the experimental infection thus produced in these animals.

On the contrary, both lysol and protargol exert antileukotactic and antiphagocytic effects, and are also potent protoplasmic poisons,

and the leukocytes with which they come in contact are injured and made to degenerate. According to the extent to which these harmful properties are exerted, the chemicals promote the advance rather than restrain the progress of meningococcic infection.

Recovery from meningococcic infection in man and animals is accomplished chiefly through the process of phagocytosis. The specific antiserum acts curatively by increasing the emigration of leukocytes, by promoting phagocytosis directly, and by agglutinating the meningococci, and also by neutralizing endotoxin. Any means which interfere with and reduce these essential processes retard or prevent recovery. Both lysol and protargol interfere with and diminish the emigration of leukocytes and the phagocytosis of meningococci, and neither possesses antitoxic power.

The mixture of antiserum with lysol and with protargol reduces to a certain extent the antileukotactic and antiphagocytic effect of the chemicals; but this action is insufficient to set aside wholly the injurious effects which they produce.

It follows, therefore, that whatever theoretical advantages might accrue from a bactericidal activity exerted by these chemicals independently of the type of meningococcus causing epidemic meningitis, is more than offset by the harmful effects which they cause.

Hence specific antiserum seems to provide the logical therapeutic agent with which to combat epidemic meningitis, since it is itself innocuous and promotes those processes essential to recovery from the disease. The problem up to the present has been that of producing an antiserum which represents the several types of the meningococcus, and this problem is now in a fair way to being solved.[1]

EXPERIMENTS ON THE DEVELOPMENT OF MALARIA PARASITES IN THREE AMERICAN SPECIES OF ANOPHELES.

By W. V. KING, Ph.D.

(*From the Bureau of Entomology, U. S. Department of Agriculture, Washington, D. C., and the Laboratories of Clinical Medicine of the School of Medicine of Tulane University, New Orleans.*)

Plates 98 to 105.

(Received for publication, March 20, 1916.)

The early results of experiments to determine the susceptibility of *Anopheles punctipennis* to infection with the parasites of tertian malaria have been recorded in two recent articles by the writer (1915–1916). In these it was shown that this species is an efficient host of *Plasmodium vivax*, and in the second article a foot-note was appended stating that it had also proven to be a host of *Plasmodium falciparum*, the parasite of estivo-autumnal malaria.

In the present paper are given further details of this series of experiments in which the susceptibility of *Anopheles crucians*, as well as of *Anopheles punctipennis*, has been established. The information acquired upon the relative susceptibility of these two species together with *Anopheles quadrimaculatus* is also included.

A study of the literature applying to this subject has revealed the fact that an extremely small amount of experimental work has actually been done with the species of *Anopheles* occurring in the United States. The experiments with *Anopheles punctipennis* were contradictory and inconclusive, and, as stated in the previous articles, the impression had become current that it was not a factor in the transmission of malaria. The proof of its efficiency as a host of malaria parasites does not, of course, establish the degree of its importance in the transmission of malaria. This depends as well upon its food habits in relation to man, its prevalence, and distribution.

In regard to *A. crucians*, nothing has been found in the early literature which establishes with certainty the proof of its susceptibility, although it has been included among the efficient hosts by several authors, notably Howard, Dyar, and Knab (1912), and Knab (1913). They refer to the experiments reported by Beyer, Pothier, Couret, and Lemann (1902), as Dupree (1905) undoubtedly does also in accrediting the proof to Couret and Beyer. The statements are evidently based upon a misinterpretation of the report.

The results of the experiments by Beyer and his associates may be summarized as follows: With *A. crucians*, no infections were obtained with tertian parasites in two specimens or with quartan parasites in three specimens, while none were tested with estivo-autumnal malaria. With *A. quadrimaculatus*, one specimen of three became infected with tertian malaria and two specimens of five with quartan. The five fed on blood containing estivo-autumnal parasites were negative. Their incrimination of *A. crucians* as a host of estivo-autumnal malaria was based on epidemiologic considerations.

Mitchell (1907) made the statement that Dupree had shown her the parasites in the salivary glands of three species (*A. quadrimaculatus*, *A. crucians*, and *A. punctipennis*). Dupree himself, however, made no reference to his having obtained infected specimens of *quadrimaculatus* or *crucians*.

Recently Mitzmain (1916) obtained negative results with 219 *A. punctipennis* dissected from 3 to 38 days after multiple bites on individuals whose blood contained varying numbers of estivo-autumnal crescents. One specimen of three *A. crucians* fed on one of the gamete carriers showed an infection.

The susceptibility of *A. quadrimaculatus* was established by the work of Thayer (1900), who obtained positive results with this species for both estivo-autumnal and tertian malaria—(one infected specimen with the former and two with the latter). Woldert (1901) conducted nine experiments with *A. quadrimaculatus*. The first experiment with tertian malaria failed. Fourteen specimens dissected in the following six experiments with tertian were all negative. In the last two, with estivo-autumnal malaria, two positive specimens were found among the seven dissected. Berkeley (1901) succeeded after repeated experiments in inoculating *A. quadrimaculatus* with the tertian parasite. Hirshberg (1904) obtained eight infected *A. quadrimaculatus* in a series of forty-eight fed on five estivo-autumnal gamete carriers. Beyer, Pothier, Couret, and Lemann reported infections in this species after the ingestion of the parasites of tertian and quartan malaria.

A fourth species of *Anopheles* (*A. pseudopunctipennis*), reported from the Southern United States, was found positive for the parasite of estivo-autumnal malaria in experiments by Darling (1910) in the Panama Canal Zone. Out of thirty-one specimens dissected, four were found to be infected. From the small number infected under the most favorable artificial conditions, and because of the fact that relatively few of this species are taken in dwellings, Darling concludes that *pseudopunctipennis* is only slightly concerned in the transmission of malaria.

The importance of the accurate determination of the exact relation of each species of *Anopheles* to malaria transmission has been emphasized by the results of a number of noteworthy investigations, which have shown that the greatest variation in susceptibility exists among the different species of this genus. Some species are entirely immune, while certain species manifest a difference in susceptibility to the different species of malaria plasmodia. Walker and Barber (1914) have given an excellent discussion of these phases of the subject in the introduction to their paper on "Malaria in the Philippine Islands."

EXPERIMENTAL.

The experiments reported below were conducted in New Orleans during the months of November and December, 1915, and January, 1916. The adult mosquitoes of the two species, *Anopheles quadrimaculatus* and *Anopheles punctipennis* were bred from larval material supplied by Mr. D. L. Van Dine, of the Bureau of Entomology, from Mound, La. Since no bred material of *Anopheles crucians* was available, specimens collected in the open were used. A flight of this species into the city occurred at this time and upon examination it was found that only a very small proportion of them had had blood meals. The specimens used for infecting purposes were selected from the ones in which no distention of the abdomen with blood or developed ova could be detected.

The experimental mosquitoes were given but one meal of infected blood. They were fed individually, not in lots as is usually done, so that the fact of their having fed was thus made certain in each case. Before the blood meal and afterwards, the diet consisted of raisins and water. Between the times of the blood meal and dissection, each specimen was kept in a separate container.

In Table I is given a part of the data obtained from the examinations of females of *Anopheles punctipennis* after they had fed on estivo-autumnal gamete carriers. The examinations were made at intervals ranging from 7 to 46 days. In all, twenty-two specimens were fed on two patients. Two specimens were not examined. On one of the patients four feedings were made on different days. Case 514 showed a medium number of gametes in the blood, but since no other species besides *punctipennis* were fed on this case, a satisfactory explanation of the negative results cannot be made.

TABLE I.

Results of Experiments with Anopheles punctipennis and Estivo-Autumnal Parasites.

Specimen No.	Date of feeding.	Date of dissection.	Interval.	Result of examination.		Case No.
				Midgut.	Salivary glands.	
	1915	*1915*	*days*			
602.1	Nov. 13	Dec. 30	47	−	+	511
602.2	" 13	" 15	32	−	−	511
617.1	" 13	Nov. 25	12	−	Not examined.	511
617.2	" 13	—				511
617.3	" 13	Dec. 6	23	−	−	511
618.1	" 13	" 29	46	−	−	511
618.2	" 13	" 27	44	−	−	511
618.3	" 13	" 27	44	−	−	511
612.6	" 22	" 21	29	−	−	511.5
626.1	" 22	" 21	29	+	+	511.5
617.4	" 23	" 14	21	+	+	511.6
618.5	" 23	Nov. 30	7	−	−	511.6
620.1	" 23	Dec. 15	22	+	−	511.6
		1916				
601.1	Dec. 23	Jan. 17	25	−	−	514
601.2	" 23	" 14	22	−	−	514
601.3	" 23	−				514
601.4	" 23	" 17	25	−	−	514
604.1	" 23	" 15	23	−	−	514
606.1	" 23	" 15	23	−	−	514
607.1	" 23	" 14	22	−	−	514
607.2	" 23	" 14	22	−	−	514
610.1	" 23	" 17	25	−	−	514

Total No. dissected.. 20
" " infected.. 4
Per cent " 20
" " " of those fed on Case 511 only.................. 33

In Specimen 602.1, the salivary glands contained only a very few sporozoites. In No. 626.1, one oocyst and one empty capsule were found upon the stomach, and the salivary glands contained large numbers of sporozoites in all the lobes of both sets. In No. 617.4 (Figs. 7, 8, and 14), one oocyst and one empty capsule were present on the stomach. The oocyst showed well developed sporoblasts. The salivary glands of this specimen were heavily infected with

sporozoites. In No. 620.1, eight oocysts were counted on the stomach, five of which were placed toward the anterior end of the enlargement of the midgut. Two of the oocysts were in the last stage of development before the release of the sporozoites, and four were large but without visible sporoblasts. The measurement of the latter gave: 47 by 48, 31 by 37, 35 by 40, and 48 by 55 microns. The other two were very small—not over 20 microns in diameter.

In Table II are shown the results of the examination of females of *Anopheles punctipennis* after feeding on tertian gamete carriers. Further details of this series have been given in a previous article (King, 1916).

TABLE II.

Results of Experiments with Anopheles punctipennis and Tertian Parasites.

Specimen No.	Date of feeding.	Date of dissection.	Interval.	Result of examination.		Case No.
				Midgut.	Salivary glands.	
	1915	*1915*	*days*			
612.1*	Nov. 6	Nov. 24	18	+	–	509
612.2	" 6	" 15	9	+	Not examined.	509
612.3	" 6	Dec. 1	25	–	–	509
614.1†	" 6	Nov. 26	20	+	+	509
614.2	" 6	" 13	7	+	Not examined.	509
614.3	" 6	" 13	7	+	" "	509
612.5‡	" 12	Dec. 2	20	+	+	510

Total No. dissected.. 7
" " infected.. 6
Per cent " 85

* Figs. 2, 3, and 4. † Figs. 9 and 11. ‡ Fig. 10.

Table III shows the results obtained with specimens of *Anopheles crucians* fed upon an estivo-autumnal gamete carrier. A total of thirty-five females were fed during a period of 5 days, but this species lived poorly in captivity, and sixteen which died soon after the blood meal were unsuitable for examination. The fact that they were "wild" mosquitoes and not bred, as were the other two

species, may account for this high mortality. Seven other specimens which were used in another experiment could not be included in the tabulation.

TABLE III.

Results of Experiments with Anopheles crucians and Estivo-Autumnal Parasites.

Specimen No.	Date of feeding.	Date of dissection.	Interval.	Result of examination.		Case No.
				Midgut.	Salivary glands.	
	1915	*1915*	*days*			
703.1	Nov. 23	Dec. 7	14	−	−	511.6
703.2	" 23	" 16	23	−	−	511.6
703.4	" 23	" 3	10	−	Not examined.	511.6
703.6	" 23	" 13	20	+	−	511.6
703.8	" 23	" 16	23	−	+	511.6
703.9	" 23	" 20	27	−	+	511.6
703.12	" 23	" 9	16	+	Not examined.	511.6
705.8	" 24	" 24	30	−	+	511.7
709.1	" 27	" 27	30	+	+ (?)	511.9
710.2	" 27	" 21	24	+	−	511.9
710.3	" 27	" 26	29	+	+ (?)	511.9
710.4	" 27	" 27	30	+	−	511.9

Total No. dissected.. 12
" " infected.. 9
Per cent " 75

Specimen 703.6 (Figs. 1, 5, and 6), had a very large number of oocysts on the stomach; the number was estimated at 75 after 39 had been counted. These ranged in size from 24 to 50 microns in diameter, the majority probably between 40 and 50. No. 703.8 had a medium infection of the center lobes of the salivary glands (compare Fig. 12). A heavy infection of all the lobes of the glands existed in No. 703.9. Five oocysts were present on the stomach of No. 703.12. Two of these measured 34 by 37 microns. In No. 705.8 a small number of sporozoites were found in the salivary glands. In No. 709.1, one large oocyst, 50 by 60 microns, was present on the stomach. The condition of the salivary glands was such that the presence of sporozoites could not be definitely ascertained. One oocyst measuring 45 microns in diameter was found on the stomach

of No. 710.2. In No. 710.3, one oocyst was present on the stomach and the glands seemed to contain sporozoites, but their condition made the diagnosis uncertain. In No. 710.4, one oocyst was seen on the stomach.

Table IV shows the infections occurring in specimens of *Anopheles quadrimaculatus* with tertian parasites, and Table V with estivo-autumnal parasites.

TABLE IV.

Results of Experiments with Anopheles quadrimaculatus and Tertian Parasites.

Specimen No.	Date of feeding.	Date of dissection.	Interval.	Result of examination.		Case No.
				Midgut.	Salivary glands.	
	1915	*1915*	*days*			
349.2	Nov. 2	Nov. 11	9	−	Not examined.	507
351.3	" 2	" 6	4	−	" "	507
352.7	" 2	" 10	8	−	" "	507
352.8	" 2	" 9	7	+	" "	507
352.9	" 2	" 12	10	+	" "	507
365.1*	" 6	Dec. 7	31	+	+	509
365.2	" 6	−				509
372.1	" 6	−				
373.1	" 6	Nov. 16	10	+	−	509
373.2	" 6	" 19	13	+	Not examined.	509
373.3	" 6	−				
347.6	" 12	Dec. 9	27	−	+	510
365.5†	" 12	" 9	27	−	+	510
365.6	" 12	" 13	31	−	−	510
371.1	" 12	−				
373.4	" 12	−				
373.5‡	" 12	Nov. 30	18	−	+	510
374.1	" 12	−				

Total No. dissected.. 12
" " infected.. 8
Per cent " 66

* Figs. 16 and 17. † Fig. 13. ‡ Fig. 15.

TABLE V.

Results of Experiments with Anopheles quadrimaculatus and Estivo-Autumnal Parasites.

Specimen No.	Date of feeding.	Date of dissection.	Interval.	Result of examination.		Case No.
				Midgut.	Salivary glands.	
	1915	*1915*	*days*			
349.1	Nov. 2	Nov. 13	11	–	Not examined.	506
354.1	" 2	" 10	8	–	" "	506
358.1	" 2	" 15	13	–	" "	506
358.2	" 2	" 15	13	–	" "	506
359.1	" 2	–				506
359.2	" 2	" 8	6	–	" "	506
359.3	" 2	" 8	6	–	" "	506
347.7	" 13	Dec. 6	23	–	–	511
350.1	" 13	" 1	18	–	–	511
350.2	" 13	–				
350.3	" 13	" 29	46	–	–	511
350.4	" 13	" 18	35	–	+	511
365.7	" 13	" 28	45	+	–	511
371.2	" 13	Nov. 20	7	–	Not examined.	511
380.1	" 22	–			–	511.5
386.1	" 23	Dec. 14	21	–	–	511.6
390.1	" 23	" 14	21	+	–	511.6
381.1	" 24	" 21	27	–	–	511.7
803.1	" 24	" 15	21	–	–	511.7
381.2	" 26	–				511.8
394.1	" 27	" 22	25	–	–	511.9
396.1	" 29	" 13	14	–	–	511.10
396.2	" 29	" 22	23	–	–	511.10

Total No. dissected.. 19
" " infected.. 3
Per cent " 15
" " " of those fed on Case 511.................... 23

Since no infections resulted from Case 506, the percentage from Case 511 only is shown.

The total number of mosquitoes of the three species examined is too small to permit of very satisfactory comparisons, but the indications of relative susceptibility as shown in Tables VI and VII are of interest. In these only those specimens which were fed on the same gamete carriers have been included. Since the females of

Anopheles crucians were not bred, while those of the other two species were, the results are perhaps not strictly comparable.

TABLE VI.

Comparative Results with Plasmodium vivax.

	A. punctipennis.	*A. quadrimaculatus.*
Total No. dissected	7	7
" " infected	6	6
Per cent "	85	85

TABLE VII.

Comparative Results with Plasmodium falciparum.

	A. punctipennis.	*A. quadrimaculatus.*	*A. crucians.*
Total No. dissected	12	13	12
" " infected	4	3	9
Per cent "	33	23	75

In Table VIII are shown the proportion of gametes to leukocytes in the blood upon which the mosquitoes fed. Case 511 was employed for feeding purposes on several different days extending over a period of 2 weeks. As may be seen from the earlier counts, the numbers of gametes were extremely high.

TABLE VIII.

Comparative Counts of Leukocytes and Gametes Made from Stained Blood Smears Taken at the Time of the Feeding of the Mosquitoes.

Case No.	Species of *Plasmodium.*	No. of leukocytes counted.	Gametes.	Gametes per 100 leukocytes.
509	*P. vivax.*	131	19	14
511	" *falciparum.*	57	300	526
511.5	" "	100	143	143
511.6	" "	200	187	93
511.7	" "	125	171	136
511.8	" "	350	106	30
511.9	" "	350	128	36
511.10	" "	300	56	18
514	" "	281	20	7

The mosquitoes used in the experiments were kept in a darkened, screened box in the laboratory. Temperature and humidity records for the entire period were kept by means of Friez recording instruments placed beside the box. The temperature of this room was usually higher and fluctuated less than the outdoor temperature, and during part of the time the room was artificially heated during the day. The temperature in the laboratory rarely fell below 60°F. On Dec. 28, however, the minimum was 51°, and on the 29th, 49°.

TABLE IX.

Weekly Mean Temperature and Per Cent Humidity (Relative) from November 1, 1915, to January 16, 1916.

Week.	Mean temperature.	Average relative humidity.
	°F.	*per cent*
Nov. 1–7	76.7	60.3
" 8–14	79.7	65.5
" 15–21	71.6	48.7
" 22–28	73.5	57.6
" 29–Dec. 5	68.5	48.8
Dec. 6–12	72	64.0
" 13–19	68.7	62.0
" 20–26	65.4	54.2
" 27–Jan. 2	64.7	72.2
Jan. 3–9	75	64.4
" 10–16	69.1	51.6

An explanation of the long developmental period of the parasites, as exhibited in these experiments, is undoubtedly found in the temperature conditions prevailing during the time. The length of the sexual cycle is usually given as from 9 to 12 days, but the exact relation of temperature to the period of development has not been carefully ascertained.

SUMMARY.

Since a knowledge of the susceptibility of any species of *Anopheles* to infection with malaria parasites is of great importance in determining its part in the transmission of malaria, the experiments reported here were undertaken, and included the three most prevalent species of this genus occurring in the United States. As a result of these experiments *Anopheles punctipennis* is shown to be an efficient

host of the organisms of tertian and estivo-autumnal malaria, *Anopheles crucians* of estivo-autumnal malaria, at least, and information has been obtained upon the relative susceptibility of these two species and *Anopheles quadrimaculatus*. The latter species has been known to be an efficient host since Thayer's experiments in 1900, and has been considered to be the principal species concerned in the transmission of malaria in the United States.

With *Anopheles punctipennis*, developmental forms of the exogenous or sporogenic cycle of *Plasmodium vivax* were demonstrated in six (85 per cent) of the seven mosquitoes dissected, and the development of *Plasmodium falciparum*, in four (20 per cent) of twenty specimens. These four infections, however, occurred in a series of thirteen specimens fed on one person, so that the percentage was actually 33.

With *Anopheles crucians*, oocysts or sporozoites or both oocysts and sporozoites of *Plasmodium falciparum* were found in nine (75 per cent) of the twelve specimens dissected. No tests were made with this species and *Plasmodium vivax*.

Anopheles quadrimaculatus was employed as a control species in the experiments and became infected in the following ratio: eight (66 per cent) of twelve specimens with *Plasmodium vivax*, and three (15 per cent) of nineteen specimens with *Plasmodium falciparum*.

In determining the relative susceptibility of the three species only those individuals which had fed upon the same gamete carriers are considered. The number of mosquitoes from which the percentages are computed is too small to make the results entirely conclusive, but the indications are that *Anopheles punctipennis* and *Anopheles quadrimaculatus* are equally susceptible to infection with *Plasmodium vivax*, 85 per cent of each species under the same conditions being positive. With *Plasmodium falciparum*, *Anopheles crucians* showed the highest percentage of infection (75 per cent), *Anopheles punctipennis* second (33 per cent), and *Anopheles quadrimaculatus* third (23 per cent).

The writer desires to acknowledge the cooperation and advice received from Dr. C. C. Bass and Dr. F. M. Johns, of the Laboratories of Clinical Medicine of the School of Medicine of Tulane University.

BIBLIOGRAPHY.

Berkeley, W. N., Some Further Work on the Mosquito-Malaria Theory, with Special Reference to Conditions around New York, *Med. Rec.*, 1901, lix, 124-128.

Beyer, G. E., Pothier, O. L., Couret, M., and Lemann, I. I., Bionomics, Experimental Investigations with *Bacillus sanarelli* and Experimental Investigations with Malaria, *New Orleans Med. and Surg. Jour.*, 1901-02, liv, 419-480.

Darling, S. T., Studies in Relation to Malaria, *U. S. Government Printer*, Washington, 1910.

Dupree, W. H., The Mosquitoes of Louisiana and Their Pathogenic Possibilities with Remarks upon Their Extermination, *New Orleans Med. and Surg. Jour.*, 1905-06, lviii, 1-16.

Hirshberg, L. K., An *Anopheles* Mosquito Which Does Not Transmit Malaria. *Bull. Johns Hopkins Hosp.*, 1904, xv, 53-56.

Howard, L. O., Dyar, H. G., and Knab, F., The Mosquitoes of North and Central America and the West Indies, *Carnegie Institution of Washington*, 1912, No. 159, i, 202.

King, W. V., The rôle of *Anopheles punctipennis* Say in the Transmission of Malaria, *Science*, 1915, xlii, 873-874.

King, W. V., *Anopheles punctipennis*, a Host of Tertian Malaria, *Am. Jour. Trop. Dis. and Prev. Med.*, 1916, iii, 426-432.

Knab, F., The Species of *Anopheles* That Transmit Human Malaria, *Am. Jour. Trop. Dis. and Prev. Med.*, 1913, i, 33-43.

Mitchell, E. G., Mosquito Life; the Habits and Life Cycles of the Known Mosquitoes of the United States; Methods for Their Control; and Keys for Easy Identification of the Species in Their Various Stages, New York and London, 1907, 136.

Mitzmain, M., *Anopheles punctipennis* Say. Its Relation to the Transmission of Malaria.—Report of Experimental Data Relative to Subterranean Malaria Fever, *Public Health Reports*, 1916, xi, 301-307.

Thayer, W. S., On Recent Advances in Our Knowledge Concerning the Etiology of Malarial Fever, *Philadelphia Med. Jour.*, 1900, v, 1046-1048.

Walker, E. L., and Barber, M. A., Malaria in the Philippine Islands., I., *Philippine Jour. Sc.*, B, 1914, ix, 381-439.

Woldert, A., Cultivation of the Estivo-Autumnal Malarial Parasite in the Mosquito—*Anopheles quadrimaculata*, *Jour. Am. Med. Assn.*, 1901, xxxvi, 559-563.

Woldert, A., Original Specimens of Zygotes of Estivo-Autumnal Malarial Parasites in the Middle Intestine of the Mosquito (*Anopheles quadrimaculatus*), *Proc. Path. Soc., Philadelphia*, 1901, N.S., iv, 129-134.

EXPLANATION OF PLATES.[1]

Plate 98.

Fig. 1. Oocysts of *Plasmodium falciparum* in *Anopheles crucians* (No. 703.6).
Fig. 2. Oocysts of *Plasmodium vivax* in *Anopheles punctipennis* (No. 612.1).

Plate 99.

Fig. 3. Two oocysts of *Plasmodium vivax* in *Anopheles punctipennis* (No. 612.1).

Fig. 4. Five oocysts of *Plasmodium vivax* in *Anopheles punctipennis* (No. 612.1).

Plate 100.

Fig. 5. Eight oocysts of *Plasmodium falciparum* in *Anopheles crucians* (No. 703.6).

Fig. 6. Ten oocysts of *Plasmodium falciparum* in *Anopheles crucians* (No. 703.6).

Plate 101.

Fig. 7. An oocyst of *Plasmodium falciparum* in *Anopheles punctipennis* (No. 617.4), showing formation of sporoblasts.

Fig. 8. Same as Fig. 7, more highly magnified.

Plate 102.

Fig. 9. An oocyst of *Plasmodium vivax* in *Anopheles punctipennis* (No. 614.1). This body contained active sporozoites and was at the point of rupturing.

Fig. 10. The empty capsule of an oocyst of *Plasmodium vivax* in *Anopheles punctipennis* (No. 612.5), after the escape of the sporozoites.

Fig. 11. Two empty capsules of oocysts of *Plasmodium vivax* in *Anopheles punctipennis* (No. 614.1).

Plate 103.

Fig. 12. A normal salivary gland of *Anopheles crucians*, showing the relation of the three lobes.

Fig. 13. A mass of sporozoites of *Plasmodium vivax* in *Anopheles quadrimaculatus* (No. 365.5). These were expelled from the salivary glands by the pressure of the cover glass.

---

[1] The author is greatly indebted to Dr. F. M. Johns for aid in the preparation of the microphotographs of infected mosquitoes, and to Dr. W. S. Thayer of Baltimore for confirmation of his interpretation of several of the preparations.

Plate 104.

Fig. 14. Sporozoites of *Plasmodium falciparum* in glands of *Anopheles punctipennis* (No. 617.4). (Although a very poor illustration it has been included since it is the only microphotograph obtained of sporozoites in this species of mosquito.)

Fig. 15. Sporozoites of *Plasmodium vivax* in *Anopheles quadrimaculatus* (No. 373.5).

Plate 105.

Fig. 16. Sporozoites of *Plasmodium vivax* in *Anopheles quadrimaculatus* (No. 365.1). This illustrates the typical clumping in one of the gland cells.

Fig. 17. Same as Fig. 16, more highly magnified.

INTESTINAL OBSTRUCTION.

VI. A Study of Non-Coagulable Nitrogen of the Blood.

By J. V. COOKE, M.D., F. H. RODENBAUGH, M.D., and G. H. WHIPPLE, M.D.

(From the George Williams Hooper Foundation for Medical Research, and the Department of Pathology of the University of California Medical School, San Francisco.)

(Received for publication, March 3, 1916.)

This communication deals with analyses of the blood in intestinal obstruction, intestinal closed loops, and other acute intoxications. The tables give figures for non-coagulable nitrogen, urea nitrogen, and in some instances the total nitrogen partition in the blood. Our interest in this study of the blood was aroused by a communication of Tileston and Comfort (1), who in a large series of human cases reported three cases of intestinal obstruction with very high non-coagulable nitrogen. Since that time we have studied the blood of various experimental animals which were being observed in connection with other experimental work.

We found that most cases of intestinal obstruction, especially with signs of acute intoxication, showed a high non-coagulable blood nitrogen, and it seemed possible that this factor might be of value in diagnosis and especially prognosis of acute abdominal conditions. We are now convinced that this non-coagulable nitrogen determination is of value in various acute intoxications. If the reading is high, one may assume a dangerous grade of intoxication, but, on the contrary, one may not assume that a low reading gives evidence of slight intoxication, because a fatal outcome may be associated with a low reading. It is of considerable value to know that the non-coagulable nitrogen of the blood may show high readings in other conditions besides renal disease.

On the other hand, determinations of the blood urea alone are of somewhat less value in studying the retention products in the blood in these conditions. In our experimental animals the blood urea

has varied from less than 30 per cent to more than 80 per cent of the total non-coagulable nitrogen, and, while a high urea reading is the rule, the variations in the urea curve and the curves of the other non-coagulable nitrogenous substances are so great that the urea reading is a somewhat unreliable index of the extent to which non-coagulable nitrogenous substances have accumulated.

Our results are given below in the form of tables and some clinical and experimental data are given in addition, but it is not needful to give the entire experimental data at this time. Usually one experiment in each table is given in some detail as an example of the group.

Methods.

Dogs were used in most experiments. A few cats were used, and one human case was included. All operations on animals were done under surgical anesthesia with the usual surgical technique.

The blood was taken in a glass syringe from the jugular vein and used for the various tests. When the blood was obtained at the time the animal was killed, it flowed from a cannula in the carotid. A few samples taken *in extremis* were obtained after anesthesia by opening the thorax and aspirating direct from the heart.

Non-coagulable nitrogen is determined as follows: With a volumetric pipette 10 cc. of blood are added to 65 cc. of distilled water, and 5 cc. of a 1 per cent sodium oxalate solution. The mixture is brought to a boil, gently rotating the flask while heating, and is then faintly acidified with acetic acid. A few crystals of sodium sulphate are added, the mixture is shaken, and, after the addition of 20 cc. of a 1 per cent solution of uranium acetate, is thoroughly shaken again and filtered. Nitrogen estimations by the Kjeldahl method are done in duplicate on 30 cc. samples of the clear filtrate,—each representing 3 cc. of blood,—and the non-coagulable nitrogen calculated for 100 cc. Creatinine and creatine are determined by the method of Folin (2) with a standard of creatinine zinc chloride. Urea is determined by the method of Marshall as modified by Van Slyke and Cullen (3). Uric acid is determined by the method of Benedict (4).

EXPERIMENTAL OBSERVATIONS.

Simple Obstruction, Recovery, and Second Operation.

Dog 15-12.—Mongrel, female; weight 40 pounds.

Mar. 8. A simple obstruction was made in the middle of the small intestine, and a small piece of tape was fixed about the small intestine to occlude its lumen but not to injure the intestinal wall.

Mar. 9. Dog quiet. Temperature 38.5°C. Slight vomiting.

Mar. 10. Dog shows usual picture of obstruction with vomiting. Temperature 38°C. Weight 38 pounds.

Mar. 11. Condition the same. Intoxication not striking. Weight 36 pounds.

Mar. 12. Condition the same. Weight 34 pounds. Late in the day the dog showed intoxication and small pulse. Infusion of 1,000 cc. of Locke's solution given in the jugular. Blood taken before and after infusion. The infusion caused clinical improvement.

Mar. 13. Dog still vomiting; the intoxication is not severe. Temperature 38.5°C. Weight 35.5 pounds. The marked drop in non-coagulable nitrogen is a striking feature of this experiment, and is difficult to explain, although the diuresis and replacement of lost fluid may be important factors. 1 p.m. Ether anesthesia and laparotomy. The obstruction was removed easily, and the intestinal wall closed over by sutures. At the end of the operation intravenous infusion of 1,000 cc. of Locke's solution. Dog made good recovery from operation.

Mar. 14. Dog vomited a little and refused food.

Mar. 15. Vomiting continues, and animal eats a little. Temperature 38.7°C. Weight 34 pounds. Given 1,000 cc. of Locke's solution intravenously.

Mar. 16. Much improvement; dog takes food; no vomiting. Weight 35 pounds.

Mar. 17 and 18. Continued improvement.

Mar. 19. Improvement continues; dog has passed feces.

Apr. 8. Dog appears normal. Second operation to establish a drained loop of duodenum including about 8 inches of the first part of the jejunum; distal portion of loop pulled through a puncture wound in left rectus; duodenum closed below pancreatic duct and gastrojejunostomy done.

Apr. 9. Dog sick, vomiting. Pulse weak. 3 p.m. Severe intoxication; much chocolate colored vomitus. Ether anesthesia. Killed.

Autopsy.—Thorax, heart, and lungs normal; abdominal viscera are normal; peritoneum is clean; gastro-enterostomy is patent; stomach shows slight engorgement of mucosa. Duodenum between pylorus and point of section shows a swollen and congested mucosa. It contains a blood-stained fluid. Jejunum below gastro-enterostomy shows engorgement of its mucosa very like that noted after lethal injection of a toxic proteose. The ileum and colon show congestion of their mucosæ to a less extent. The loop contains chocolate-red, slimy material; the mucosa shows no ulceration, but is congested and deep red; there are some submucous ecchymoses.

This experiment is of interest because of the recovery from the first obstruction. An obstruction was produced in the middle of the small intestine by means of a tape. On the 6th day the tape was removed at a second operation, and the dog returned to a normal

TABLE I.

Dog 15–12. Simple Obstruction, Recovery, and Second Operation.

Day after operation.	Time.	Non-coagulable nitrogen.*	Remarks. Mongrel, female; weight 40 lbs.
		mg.	
2	2.00	23	Vomited once. Pulse good. Temp. 38.5°C.
3	11.00	24	Dog vomiting. Condition good. 38 lbs.
4	4.00	68	Dog vomiting frequently. 36 lbs. Temp. 38°C.
5	11.00	119	Dog vomiting. Pulse good. Feces in cage. 34 lbs. Temp. 38°C.
5	2.50	131	Dog prostrated. Pulse tension low. Infusion of 1,000 cc. of Locke's solution.
5	3.20	117	Blood after infusion. Clinical improvement.
6	11.30	31	Dog improved. Vomiting less.
2nd operation.	1.00	42	Laparotomy and removal of obstruction. Infusion of 1,000 cc. of Locke's solution. Blood at end of infusion.
7	11.00	36	Vomits occasionally. Refuses food.
9	10.00	26	Dog improving. 35 lbs.
11	4.00	28	Dog improving rapidly. 35.5 lbs. Temp. 38.4°C.
12	11.00	31	Dog passed feces. Seems quite well.
32	—	—	Normal.
3rd operation.	3.00	—	Drained loop of jejunum. Long operation.
33	12.00	56	Dog much intoxicated and vomiting. Urea nitrogen 31 mg.
33	3.00	71	Animal acutely sick. Killed. Amino nitrogen 9.3 mg.

* Non-coagulable nitrogen is given in terms of mg. per 100 cc. of blood.

condition. The third operation was performed, and about 18 inches of the lower duodenum and first portion of the jejunum were isolated as a drained loop opening in the left side. A gastro-enterostomy was performed. The animal showed grave intoxication with much vomiting and death in 24 hours. There was no peritonitis but some interference with the blood supply of the drained loop.

Table I shows the fluctuation in the blood non-coagulable nitrogen. There was a steady rise to a maximum of 131 mg. per 100 cc. of blood on the 5th day. The dog was given an infusion, and the following day the non-coagulable nitrogen had dropped to 31 mg. One is tempted to assume as a simple explanation that the replace-

ment of fluid and the consecutive diuresis sweep out these substances from the blood. This undoubtedly does occur, but the story is not quite as simple as this. For example, in other experiments with a grave intoxication a transfusion may not affect in the least the level of blood non-coagulable nitrogen, in spite of a striking diuresis. There is no evidence to point to lack of eliminative power of the kidneys as a factor.

TABLE II.

Simple Obstruction.

Dog No.	Day after operation.	Non-coagulable nitrogen.*	Urea nitrogen.*	Weight.	Temp.	Remarks.
		mg.	*mg.*	*lbs.*	*°C.*	
15–20	6	48	—	15.3	38.5	No vomitus. Dog quiet.
	7	33	—	14.5	38.4	Condition the same.
	8	38	—	14.3	39.1	" " "
	9	40	19	13.8	38.5	Little vomitus, intoxication moderate. Killed.
15–26	1	77	—	—	—	Blood after operation.
	3	46	34	42.8	39.2	Vomiting and sick.
	4	60	35	41.3	39.2	Condition the same.
	5	56	40	40.8	39.3	" " "
	6	60	39	40.0	39.1	" " "
	7	94	58	39.0	39.6	Dog weak.
	8	79	42	—	—	Dog seems better. Killed. Slight chronic nephritis.
16–45	1	34	15	36.0	—	Blood before operation.
	3	52	28	34.0	39.7	Vomiting large amounts.
	4	59	21	33.0	39.3	Vomiting.
	6	90	43	—	—	Dog acutely sick. Killed.
16–56	1	—	22	22.0	—	
	4	—	22	19.0	38.0	Dog vomiting since operation.
	5	63	21	—	37.7	11 a.m. Animal in fair condition. Proteose injection. Blood before injection.
	5	81	27	19.5	—	11.10 a.m. Recovered from proteose injection in 6 hrs. Died in night. Blood after injection.

* Non-coagulable and urea nitrogen are given in terms of mg. per 100 cc. of blood.

Simple Obstruction for 5 Days. Toxic Death.

Dog 16–45.—Shepherd, mongrel, female; weight 36 pounds.

Nov. 10. Simple obstruction in the middle of the small intestine by means of complete section and an inversion of cut ends.

Nov. 11 and 12. Dog is vomiting, and there is a steady loss in weight.

Nov. 13. Much thin yellow vomitus. Temperature 39.3°C. Weight 33 pounds.

Nov. 15. 9 a.m. Dog is severely intoxicated. Ether anesthesia. Killed. The readings for non-coagulable nitrogen and urea nitrogen are given in Table II.

Autopsy.—Thorax, lungs, and heart normal. The viscera show nothing of interest except a slight congestion. The peritoneum is dry and clean. There are a few fibrinous adhesions about the site of obstruction. Intestinal tract below obstruction is collapsed and mucosa normal. The intestine above the obstruction shows only slight dilatation. There is no congestion of the mucosa and no ulceration. The stomach shows come congestion of the cardia. The material above the obstruction consists of thin yellow material, like pea-soup. 160 cc. in amount. Kept for further study.

The protocol of Dog 16–45 gives a typical story of an uncomplicated, untreated case of simple obstruction of the middle of the small intestine. Vomiting is constant, but the grave intoxication does not appear until the 5th day, when the non-coagulable nitrogen is found to be high—90 mg. per 100 cc. of blood. The autopsy findings are characteristic, and the peritoneum is clean.

Table II shows a considerable variety of non-coagulable nitrogen readings. The last three experiments show a definite rise in non-coagulable nitrogen above normal, and this is the rule. The first experiment, however, is a good example of a dog which maintained practically a normal level in spite of a prolonged obstruction. It should be kept in mind that this dog had a resistance considerably above normal, consequently was not as acutely intoxicated, and was killed 8 days after operation while still in fair condition. A similar state of affairs is noted in closed loops of the intestine, and with a slowly progressing intoxication the non-coagulable nitrogen may scarcely rise above its normal level.

This table of simple obstruction experiments gives also the urea nitrogen of the blood. It is noted at once that these intoxications give different urea readings with high non-coaguable nitrogen than is found in chronic nephritis. Urea nitrogen in nephritis usually

constitutes more than 50 to 60 per cent of the total nitrogen, but here it is seen that it often falls below 50 per cent. The residual or undetermined nitrogen in these experiments is constantly high. We suspect that this is a fairly constant feature in various proteose intoxications.

TABLE III.

Closed Loop of Duodenum and Jejunum. Gastro-Enterostomy.

Dog. No.	Day after operation.	Non-coagulable nitrogen.*	Remarks.
		mg.	
15–2	2	41	Condition good, vomiting.
	3	89	" "
	4	113	Grave intoxication; subnormal temperature. Killed.
15–9	1	51	Blood taken at time of operation. Weight 33 lbs.
	2	103	Dog sick, listless, vomiting. Died during night.
15–10	1	20	Blood at operation. 23 lbs.
	2	35	Dog toxic. Pulse good.
	3	70	Dog in fair condition. Intoxication moderate.
	4	135	Dog toxic. Pulse weak. Killed.
15–14	1	40	Blood before operation. 30 lbs.
	2	33	Dog vomiting. Slightly toxic.
	3	37	" " Moderately toxic. 28.5 lbs.
	4	53	Little vomiting. Condition the same.
	5	55	11 a.m. Dog sick. Muscular tremors. 26.5 lbs.
	5	65	2.30 p.m. Killed. Amino nitrogen 5.7 mg. per 100 cc.

* Non-coagulable nitrogen is given in terms of mg. per 100 cc. of blood.

Long Loop of Duodenum and Jejunum. Gastro-Enterostomy.

Dog 15–10.—Long haired mongrel, male; weight 23.5 pounds.

Mar. 3. Isolated a long loop including lower half of duodenum and upper part of the jejunum; occlusion was effected by means of tapes applied as usual; gastro-enterostomy performed.

Mar. 4. Dog intoxicated. Temperature 38.5°C.

Mar. 5. Condition unchanged.

Mar. 6. Dog very sick; much vomiting. Pulse pressure low. Slow respiration. Temperature 37.3°C. Ether anesthesia and bleeding from carotid, which showed an arterial pressure scarcely above the usual venous pressure.

Autopsy.—Thorax normal. Peritoneum is clean and dry, except for a few bits of fibrin close to the site of operation. Liver shows a little congestion.

Other organs are normal. The loop is completely isolated from the intestine; it contains about 50 cc. of creamy, white, syrupy material having the characteristic odor. The mucosa shows a very slight amount of congestion, no ulceration, and is intact throughout. Under the microscope it appears normal. Loop fluid preserved for further study.

The protocol of Dog 15–10 (Table III) is a good example of uncomplicated closed intestinal loops of a certain type. It will be seen that the non-coagulable nitrogen of the blood rises regularly with the intoxication developing under these conditions. It must not be forgotten that these loops include the lower half of the duodenum and much of the upper jejunum. The ends of the loop are closed by tapes, and the contents of the upper duodenum must be forced back into the stomach where they gain access to the jejunum by a posterior gastrojejunostomy. This experiment, therefore, causes an obstruction to the first half of the duodenum, and this is of no small importance, as is seen on comparing these experiments with other closed loops of a different sort in Table V. We intend to take up this point in another communication.

TABLE IV.

Closed Loop of Jejunum. Gastro-Enterostomy.

Dog No.	Day after operation.	Non-coagulable nitrogen.*	Remarks.
		mg.	
15–29	2	23	Blood at end of infusion. Intoxication definite. Infusion of 1,000 cc. of 1% dextrose solution. Weight 35 lbs.
	4	35	Vomiting. Pulse fair.
	5	39	Dog weak. Blood taken before infusion. 1,000 cc. of 10% dextrose. 32 lbs.
	6	98	5 p.m. Infusion of 1,000 cc. of 10% dextrose. 11 p.m. Animal moribund. Killed. Loop rupture.
15–45	4	30	Dog slightly intoxicated. 22 lbs.
	5	76	10 a.m. Dog about the same.
	5	115	5 p.m. Dog looks sick. Pulse weak. Killed. Early peritonitis.
15–19	1	31	Blood at end of operation. 22 lbs.
	2	62	Dog very quiet. Died during night. Acute intoxication.

* Non-coagulable nitrogen is given in terms of mg. per 100 cc. of blood.

Long Loop of Jejunum with Rupture and Peritonitis.

Dog 15–29.—Adult spaniel, male; weight 35 pounds.

Apr. 23. Isolated a long loop including the lower portion of the duodenum and about 3 feet of the jejunum, using tapes. Gastrojejunostomy performed as usual.

Apr. 24. Dog is sick. There is much dark brown vomitus. Pulse poor, and for this reason infusion of normal salt solution plus 1 per cent dextrose was given intravenously, followed by much improvement.

Apr. 25. Intoxication not striking.

Apr. 26. Much vomiting. Weight 33 pounds. Temperature 38.5°C.

Apr. 27. Dog seems sick. Weight 32 pounds. Temperature 38.4°C. 12 m. Infusion of 1,000 cc. of 10 per cent dextrose. 5 p.m. Dog not much improved. Given a second infusion of 1,000 cc. of 10 per cent dextrose.

Apr. 28. Much vomiting. Pulse tension low. Temperature 38.4°C. Weight 31.3 pounds. 5 p.m. Infusion of 1,000 cc. of 10 per cent dextrose. Dog is vomiting constantly. 11 p.m. Dog very sick. Ether anesthesia. Killed.

Autopsy.—The peritoneal cavity contains 500 cc. of blood-tinged loop fluid of the usual appearance. The peritoneum is specked with ecchymoses, but there is no fibrin. Rupture took place at the site of the lower ligature, which cut through the wall of the intestine. Upper ligature is tight. Death took place promptly from absorption of the toxic material poured from the loop into the peritoneum. The organs show some congestion associated with intoxication by means of loop fluid. Loop is empty. It shows congestion and some areas of submucous hemorrhage due undoubtedly to acute distention.

The protocol of Dog 15–29 (Table IV) shows the same rise in non-coagulable blood nitrogen noted in the closed intestinal loop experiments of Table III. These experiments show various complications met with in these loop experiments,—peritonitis, rupture of loop, and overwhelming intoxication. When the intoxication is very acute and severe, the infusion of normal salt or dextrose solutions will not depress the level of non-coagulable nitrogen in the blood.

Long Loop of Jejunum. Rupture in 20 Days.

Dog 16–22.—Fox-terrier, male; weight 18.5 pounds.

Sept. 28. Isolated a long loop of jejunum; ends of loop sectioned and turned in; the jejunum joined around the loop by end to end anastomosis to establish direct continuity of intestinal lumen.

Sept. 29. Dog looks well; no vomiting. (See Table V for details of temperature, weight, and non-coagulable and urea nitrogen). There was steady loss of weight during the next week with occasional attacks of vomiting; at times the dog eats a little food.

TABLE V.

Long Loop, Jejunum, Ileum.

Dog No.	Day after operation.	Non-coagulable nitrogen.*	Urea nitrogen.*	Weight.	Temp.	Remarks.
		mg.	*mg.*	*lbs.*	°C.	
16–20	1	49	9	39.0	—	Before operation. Long loop jejunum.
	1	50	8	—	—	After operation.
	3	60	4	—	40.0	Condition good.
	4	28	7	—	40.0	Dog has vomited.
	5	35	11	37.5	40.1	No vomiting.
	8	24	8	36.8	40.1	" "
	9	22	6	37.3	—	Condition improved.
	10	28	8	37.5	39.8	Dog in good condition.
	11	28	8	36.5	39.7	" " " "
	13	33	12	35.3	—	Dog vomiting and intoxicated.
	14	53	16	—	—	Dog vomiting. Infusion of 1,000 cc. of 7% dextrose solution.
	15	51	18	33.0	39.3	Dog vomiting, weaker. Death 18th day.
16–22	1	22	10	18.5	—	Before operation. Long loop jejunum.
	3	36	8	18.0	39.4	Intoxicated. No vomiting.
	4	—	16	17.5	—	Vomiting, large amount.
	5	35	19	17.3	39.3	Little vomiting.
	7	30	14	15.8	38.9	" " Loss of strength.
	9	36	10	15.5	39.1	No vomiting.
	11	35	12	15.5	39.1	Solid feces.
	14	36	6	15.3	—	Condition the same.
	15	48	9	15.0	—	No vomiting.
	16	43	10	14.5	—	Animal looks intoxicated.
	20	41	20	13.7	38.8	Dog weak. Infusion of 1,000 cc. of 7% dextrose.
	21	64	29	—	—	Dog sick. Killed.
16–37	5	90	21	27.3	38.9	Vomiting for past 3 days. Long loop of ileum.
	6	45	20	27.3	39.1	Dog improved; no vomiting; eating.
	10	53	20	25.8	38.9	Some diarrhea.
	11	—	13	—	—	Dog looks well.
	13	59	21	24.3	—	Condition the same.
	14	57	25	24.0	—	Dog improved.
	15	31	19	23.8	—	Passed solid stool. Eating.
	17	54	32	24.0	38.9	Peristalsis visible in abdomen.
	18	40	19	24.0	39.3	Dog losing ground.
	19	40	9	23.8	38.9	Condition good. Killed.

* Non-coagulable and urea nitrogen are given in terms of mg. per 100 cc. of blood.

Oct. 14. Dog has passed formed stools. No vomitus. Weight 14.5 pounds. There is evidence of chronic intoxication. Dog was injected with a toxic proteose obtained from ox pancreas; dog was slightly intoxicated by this proteose, while a normal control animal was fatally poisoned in 11 hours by a similar dose.

Oct. 15. Dog looks well; no vomitus.

Oct. 18. Dog is very sick. Ether anesthesia. Killed.

Autopsy.—Thorax, heart, and lungs normal; spleen, kidney, pancreas, etc., normal; liver rather atrophic; no signs of fatty degeneration. Peritoneal cavity contains a good deal of the loop fluid which has escaped from a recent rupture. There is little reaction in the peritoneum as the intoxication was so acute. Stomach and intestine outside of loop show congestion of the mucosa so common in proteose intoxication. Loop is made up of two parts isolated by old adhesions. One-half is slightly collapsed due to escape of fluid into the peritoneum; there is an ulcer in the wall, which has perforated the mucosa; all this portion is somewhat red and swollen, and shows two other ulcers involving the mucosa. The other half of the loop consists of three small coils twisted about its mesenteric attachment; adhesions have caused some constriction of mesenteric vessels giving engorgement of the loop but not infarction; this portion of the loop contains a pale, slate colored fluid with no blood; the mucosa is swollen and engorged but shows no hemorrhage; the walls of the loop are everywhere hypertrophied.

The protocol of Dog 16–22 (Table V) shows a different type of closed intestinal loop with a slowly progressing intoxication and relatively slight changes in the non-coagulable nitrogen of the blood. The loops of the jejunum or ileum are isolated completely by cross section of the gut in two places. The loop is made by turning in the ends of the isolated portion of the intestine or by doing an end to end anastomosis thus forming a circle out of the isolated gut. The continuity of the rest of the intestine is established by means of an end to end anastomosis which gives an unobstructed flow from duodenum to jejunum and ileum. This does away with the obstruction in the first half of the duodenum which is present in the loops isolated by ligature and gastrojejunostomy (Tables III and IV). The difference in severity of intoxication under these conditions is obvious.

The urea nitrogen readings in general show a low percentage value of the total non-coagulable nitrogen. The weight curve shows the gradual loss in body weight due to the chronic intoxication, even in the absence of vomiting.

The immunity to proteose injection (Dog 16–22) shown by a dog with a long standing closed intestinal loop indicates that the presence

of a closed loop of intestine causes a chronic proteose intoxication which gives a certain degree of immunity against poisoning by various foreign proteoses. This point will be taken up in another communication and the experiments will be given in detail.

TABLE VI.

Long Loop, Jejunum, Ileum, Complications.

Dog No.	Day after operation.	Non-coagulable nitrogen.*	Urea nitrogen.*	Weight.	Temp.	Remarks.
		mg.	*mg.*	*lbs.*	*°C.*	
16–42	3	32	13	21.0	39.1	Long loop jejunum, obstruction, peritonitis.
	4	39	15	20.5	39.0	Considerable vomiting.
	5	39	13	20.7	38.8	" " Animal looks sick.
	6	50	22	21.0	37.9	9.30 a.m. No vomitus.
	6	56	39	—	34.5	4 p.m. Dog toxic. Killed.
16–48	4	30	14	19.5	39.0	Long loop jejunum, terminal obstruction, and volvulus.
	6	45	19	19.0	39.2	Condition good. No vomiting.
	8	32	15	19.3	38.8	Condition the same.
	12	46	22	19.5	39.3	" " " Found dead on 13th day.
16–57	1	58	23	18.5	—	Short loop of ileum. General peritonitis.
	6	44	11	15.5	39.3	Dog has been in good condition.
	8	166	51	15.5	36.8	Some vomiting. Animal moribund. Killed.
16–71	1	36	12	27.5	—	Long loop of jejunum. General peritonitis.
	2	36	12	26.3	38.3	Dog in fair condition.
	4	53	31	—	—	Animal moribund. Killed.
16–39	1	—	15	24.5	—	Long loop of jejunum. General peritonitis.
	3	71	18	23.5	38.8	Much vomiting. Pulse poor. Infusion of 1,000 cc. of 5% dextrose. Blood at end of infusion. Death next day.

* Non-coagulable and urea nitrogen are given in terms of mg. per 100 cc. of blood.

Long Loop of Jejunum. Obstruction. General Peritonitis.

Dog 16–42.—Mongrel, male; weight 22 pounds.

Nov. 6. Isolated a long loop of jejunum by section and inversion of ends; jejunum joined around the loop by means of end to end anastomosis between upper and lower end.

Nov. 8. Dog pretty well. Temperature 39.2°C. Weight 22 pounds.

Nov. 9. Much vomiting.

Nov. 10. Dog seems better. (See Table VI for details.)

Nov. 11. Little vomiting. Temperature 37.9°C. Weight 21 pounds. 4 p.m. Dog is very weak. Temperature 34.5°C. Ether anesthesia. Killed.

Autopsy.—Thorax, heart, and lungs normal. Peritoneal cavity contains many isolated pockets of purulent exudate. A large round worm is free in the peritoneal cavity, evidently having escaped from the loop, which contains numerous similar worms. There are organized adhesions just below the end to end anastomosis causing a sharp kink and probably complete obstruction. Duodenum and jejunum contain the usual obstruction fluid. The loop contains 130 cc. of pale, slate colored fluid with a strong odor. There are numerous live and active round worms in the loop. No point can be found where the worm escaped from the loop. The peritonitis is probably of 1 or 2 days' duration.

The protocol of Dog 16–42 (Table VI) shows some of the complications which may arise in the closed loop experiments. Peritonitis is most common, but volvulus and obstruction may occur. These complications do not modify the picture, and we have good evidence that peritonitis alone may be associated with a high non-coagulable nitrogen. It is of interest to note that we have been able to isolate a toxic proteose from peritoneal exudates, and we believe that this proteose is of considerable importance in explaining the intoxication of general peritonitis. The rise in non-coagulable nitrogen in the blood may be in part due to the proteose intoxication.

Proteose Injection. Rise in Non-Coagulable Nitrogen.

Dog 15–50.—Small black and tan, male; weight 13 pounds.

12 m. (A) Blood non-coagulable nitrogen 33 mg. per 100 cc. of blood; ether anesthesia and intravenous injection of purified proteose obtained from closed intestinal loops. Kymograph observation during slow injection of 65 cc. of solution of proteose which had been reprecipitated first by alcohol and then by half saturation with ammonium sulphate as described previously (5). There was a little fall in blood pressure.

12.45 p.m. After injection (B) blood non-coagulable nitrogen 40 mg. per 100 cc. of blood.

3.15 p.m. (C) Blood non-coagulable nitrogen 80 mg. per 100 cc. of blood.

4 p.m. Death with prostration and subnormal temperature.

Autopsy.—There is the typical splanchnic engorgement due to fatal proteose intoxication. Spleen and liver are swollen and purple. Mucosa of duodenum and jejunum is swollen and deep reddish purple.

Dog 15–51.—Small fox-terrier, female; weight 15.5 pounds.

12 m. (A) Blood non-coagulable nitrogen 39 mg. per 100 cc. of blood. Ether anesthesia and intravenous injection of purified proteose obtained from closed intestinal loops. Kymograph observation during injection of 50 cc. of fluid showed no change in blood pressure.

12.30 p.m. After injection (B) blood non-coagulable nitrogen 32 mg. per 100 cc. of blood.

5 p.m. (C) Blood non-coagulable nitrogen 58 mg. per 100 cc. of blood.

Dog is prostrated; much vomiting and diarrhea.

Died in night.

Autopsy.—The findings are characteristic of proteose intoxication. There is much blood-tinged fluid in the intestines, and the mucosa is red and swollen.

These two experiments (Dogs 15–50 and 15–51) are of considerable interest, and show that acute poisoning with a pure proteose may cause the blood non-coagulable nitrogen to double in amount in 3 hours; for example, a rise from 40 to 80 mg. The proteose used in these experiments was pure, and not over 200 mg. were injected. The addition of this to the blood itself could not be detected by any method in use, and the method used by us causes precipitation of all primary proteoses at least. The great rise in non-coagulable nitrogen obviously must be explained by disintegration of body or tissue protein. This is of importance in explaining the high non-coagulable nitrogen associated with the closed loops of intestine or intestinal obstruction. In both instances we are dealing with a proteose intoxication, and we believe that much of the increase in blood non-coagulable nitrogen is due to disintegration of the tissues of the body. Catabolism, in other words, must be responsible for much of the non-coagulable nitrogen rather than retention.

Tables VII and VIII give the nitrogen partition of the total non-protein nitrogen of the blood expressed in mg. per 100 cc. of blood. One control nephritis gives a residual nitrogen of 11 per cent and 82 per cent urea nitrogen. A second case (Dog 16–49 in Table VIII) shows a similar picture. This experiment presents isolation of the bladder and implantation of the ureters into the intestine. There

TABLE VII.

Intestinal Loops. Peritonitis and Obstruction.

No.	Non-coagulable nitrogen.	Urea nitrogen.		Amino nitrogen.	Uric acid nitrogen.	Creatine nitrogen.	Creatinine nitrogen.	Residual nitrogen.		Remarks.
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	
Dog 16–37	40	9	23	1.4	0.6	0.6	1.5	26.9	67	Long loop of ileum. Killed 19th day.
" 16–42	56	39	69	4.5	0.6	1.0	5.6	5.3	9	Long loop of jejunum. Peritonitis, obstruction.
" 16–57	166	51	30	6.0	0.6	0.8	11.0	96.6	58	Long loop of ileum. Peritonitis.
" 16–71	53	31	58	3.7	1.1	0.8	7.3	10.1	19	Long loop of jejunum. Peritonitis.
" 16–80	40	28	70	3.9	1.0	0.3	3.1	3.7	9	Long loop of ileum. Peritonitis.
" 15–45	115	77	60	—	—	0.8	5.9	—	—	Long loop of jejunum.
Cat 16–7	296	137	46	6.8	2.3	0.7	21.3	127.9	43	Long loop of jejunum and obstruction. Death in 4 days.
Dog 16–45	90	43	47	5.5	—	—	—	—	—	Simple obstruction. Death in 5 days.
" 16–82	47	17	36	2.5	1.4	0.2	3.3	22.6	48	Simple obstruction. Killed in 7 days.
Case 1 (Man)	164	107	65	2.3	0.9	0.3	10.2	35.2	21	Simple obstruction. Pneumonia. Death in 5 days.

Nitrogen is given in terms of mg. per 100 cc. of blood.

was some obstruction to the outflow of urine and some escape into the peritoneum with an irritant peritonitis and absorption consequent to this. Here there are two factors—retention or lack of elimination plus peritonitis.

The tables show a high per cent of residual nitrogen or a low relative per cent of urea nitrogen. This is true particularly in the severe proteose intoxication with high non-coagulable nitrogen. Similar high readings in cases of nephritis and retention will scarcely show such high percentages of residual nitrogen. For this reason we be-

TABLE VIII.

Bladder Isolation, Chronic Nephritis, Peritonitis, etc.

Dog No.	Non-coagulable nitrogen.	Urea nitrogen.		Amino nitrogen.	Uric acid nitrogen.	Creatine nitrogen.	Creatinine nitrogen.	Residual nitrogen.		Remarks.
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	
16–49	130	91	70	2.3	0.3	0.4	7.1	28.9	22	Bladder isolation. Urine escape. Peritonitis.
16–61	368	304	82	6.8	2.3	1.0	12.0	41.9	11	Chronic nephritis. Old dog. Hemorrhagic gastritis.
16–62	72	42	58	4.2	1.0	0.5	1.1	23.2	32	Acute distemper.
16–65	36	18	50	2.8	0.3	0.6	5.2	9.1	25	Drained loop of ileum. Subcutaneous abscess.
16–67	40	16	40	1.5	1.8	0.6	3.8	16.3	40	Skin incisions. Mild distemper.
16–69	164	58	38	4.2	1.2	0.9	5.3	94.4	57	General peritonitis.
16–85	67	29	43	3.1	0.4	0.7	3.6	30.2	45	Distemper. Pneumonia.

Nitrogen is given in terms of mg. per 100 cc. of blood.

lieve that the non-coagulable nitrogen determination gives more information than does urea nitrogen, and is of more clinical value in diagnosis and prognosis.

The blood content of amino nitrogen and uric acid nitrogen is low normal, and shows fluctuations within normal limits. Ammonia nitrogen was done in the majority of experiments but the method is inaccurate and the values are probably too high. It seemed best to leave these analyses out of the table and assume that ammonia nitrogen is included in the urea nitrogen. The difference scarcely exceeds 1 mg. at the most.

The creatine fraction is constantly low, but it is of interest to note that the creatinine nitrogen may be very high in many of these proteose intoxications, particularly in intestinal obstruction and closed intestinal loops. There are many readings of more than 5 mg. per 100 cc. of blood, and one experiment (Table VII, Cat 16–7) shows a reading of 21.3 mg. This occurred in a cat with intestinal obstruction plus a closed intestinal loop with a non-coagulable nitrogen of 296 mg. per 100 cc. of blood. The kidneys in all these cases,

unless otherwise noted, were normal. It is to be recalled that all these animals were sick and refused food—in fact the majority were vomiting more or less. A human case of intestinal obstruction and pneumonia shows a very high creatinine value, 10.2 mg., but autopsy showed practically normal kidneys.

Human Intestinal Obstruction. Death in 5 Days.

Case I.—C. K., German, male, 68 years of age.

Past History.—Negative.

Present Illness.—Began Jan. 16, 1916, with indefinite pain. No bowel movements since this time.

Jan. 17. A good deal of epigastric pain with vomiting, which became fecal in type the following day. Patient given castor oil and salts without results.

Jan. 18. Condition unchanged, except that vomiting became more severe.

Jan. 19. Entered hospital. At this time he had marked abdominal tenderness, and was vomiting frequently. Vomitus fecal in odor, watery, with fine brownish precipitate. Blood obtained showed very high non-coagulable nitrogen. Patient given 900 cc. of 6 per cent glucose with sodium carbonate intravenously. Infusion improved condition of patient.

Blood Examination.—White blood cells 18,400; hemoglobin 90 per cent.

3 p.m. Laparotomy and abdominal exploration. A portion of greatly congested intestine, about 10 cm., was found; the wall was edematous. It was placed back in the abdominal cavity. Abdomen closed. Volvulus(?).

Jan. 20. 10 a.m. Blood again obtained, and showed some decrease in non-coagulable nitrogen; vomiting not so marked; considerable fecal matter obtained by means of an enema. Pulse not good. 4 p.m. Blood again obtained; showed rising non-coagulable nitrogen. Given infusion of 750 cc. of 6 per cent glucose intravenously.

Jan. 21. 6 a.m. Patient died in stupor. Blood obtained by cardiac puncture a few minutes after death.

Autopsy performed 5 hours after death.

Anatomical Diagnosis.—Intestinal obstruction; volvulus (?); operation wound for relief of obstruction; early infarction and necrosis of loop of ileum; early serofibrinous peritonitis; bronchopneumonia (pseudolobar) of both lungs; acute hemorrhagic bronchitis; cloudy swelling of viscera.

The abdomen is considerably distended and tense. On incision a small amount of blood-tinged, slightly turbid fluid is found between the coils of an enormously distended small intestine. The loops are somewhat glued together by dry, plastic exudate. The surface of the intestines is somewhat dry and very definitely injected, more especially in the region of the abdominal incision. There are no adhesions except over the spleen. There are no hernial openings to be found in the pelvis or inguinal region. One of the stitches in the abdominal wound has caught

and firmly held a bit of omentum. One segment of the intestine, dark red in color and considerably swollen, measuring about 15 cm. in length, is found close to the liver. The swelling involves the wall of the intestine and the mesentery to a distance of about 5 cm. from the intestinal attachment. There is a clean-cut line of demarcation between the relatively normal but elongated mesentery and the short, inflamed, edematous mesenteric portion close to the intestine. The line of demarcation on the intestine is quite sharp, particularly at the upper end. It appears as though a band, or definite tight constriction, had been drawn about the portion of the ileum including the small part of the mesentery, shutting off a considerable part of the blood supply. This may have been due to a twist of the relaxed elongated mesentery. This was evidently relieved at operation, but the circulation did not establish itself properly owing to tissue injury, and the general appearance was that suggesting early hemorrhagic infarction. The picture, however, was not complete. Careful section of the mesenteric veins showed them to be quite free from thrombi even in their finer branches. There may be some very small thrombi in the smaller branches close to the mesenteric border, but these could not be dissected out.

Lungs.—The right lung weighs 870, and the left 560 gm. The lungs are voluminous, cushiony in their anterior portions; they are heavy and consolidated posteriorly. The pulmonic vessels are clear. The bronchi are intensely inflamed, their mucosa is velvety and purple, and they contain much serous blood-tinged fluid. On section the anterior portions are dry and cushiony. The posterior portions are consolidated and very moist. One can scrape off purulent material. The consolidation involves the posterior portion of the left upper and the greater portion of the right upper lobe and part of the right middle lobe. These areas of consolidation are mottled gray and purplish red. Some of the gray areas are very soft, and creamy material can be scraped off, indicating a beginning resolution of the exudate. This pneumonic process must have been of several days duration—estimated 2 to 3 days.

Kidneys.—Capsule comes off easily leaving a smooth surface, but for two retention cysts. There is one large retention cyst in the upper pole of the right kidney measuring about 2 cm. in diameter. Its wall contains a few thin plaques of calcified material. The kidney parenchyma on section appears normal. The pelvis is normal.

Microscopic Examination. Kidneys.—There are a few hyaline casts in some of the tubules, also a few hyaline scars in the cortex. In general the parenchyma looks normal except for definite cloudy swelling of the epithelium lining the convoluted tubules. There is no epithelial necrosis. The stroma of the pyramids shows a little edema.

Lungs.—The patches of pneumonia show the alveoli filled with an exudate of coagulated serum, mono- and polynuclear cells, fibrin, and enormous numbers of bacteria. In places the bacteria form almost a solid mat of rods showing capsules. The great overgrowth of bacteria is the striking feature of this lung.

Mesentery.—The swollen hemorrhagic area shows a few small recent thrombi, but most of the veins are free. There is extreme extravasation of red blood cells into its stroma. The other portion of the anatomical protocol may be omitted, as it has no bearing on the points under consideration.

TABLE IX.

Human Intestinal Obstruction.

Time.	Non-coagulable nitrogen.*	Urea nitrogen.*	Urea.	Remarks.
	mg.	*mg.*	*per cent*	
2 hrs. before operation.	145	30	20	After infusion of 900 cc. of 1% sodium carbonate and 6% glucose.
18 " after "	76	48	63	After infusion of 400 cc. of 6% glucose.
26 " " "	80	52	60	" " " 750 " " 6% "
46 " " "	164	107	65	Heart blood (see Table VII).

* Non-coagulable and urea nitrogen are given in terms of mg. per 100 cc. of blood.

The human case (Table IX) is of much interest as the observations on the blood are fairly complete, and autopsy material is available. Clinically it was a clear case of obstruction due to volvulus with partial infarct formation in the intestine and a complicating pneumonia. The non-coagulable nitrogen of the blood was constantly high in spite of transfusion at various times. The elimination of urine was considerable, because of the transfusions, and showed nothing of interest—a mere trace of albumin and an occasional cast.

Table IX shows that there is a rise in urea and drop in non-coagulable nitrogen after the first infusion. We have noted this under the same circumstances in dogs, but are not able to advance an explanation. The high creatinine value (Table VIII) is of interest, as the kidneys are shown to be practically normal. This case gives blood findings identical with those observed in animals under similar conditions, and indicates that our experiments give information which may be of value in the study of human cases.

DISCUSSION.

We believe that it may be assumed as proven that the non-coagulable nitrogen of the blood in the majority of instances is definitely

increased in intestinal obstruction or with closed loops of intestine. With acute intoxication this rise in non-coagulable nitrogen is apt to be more striking and constant. When this rise in non-coagulable nitrogen of the blood does occur, it is a grave sign, and is a clinical index of a severe intoxication even in spite of other clinical evidence to the contrary. But a low non-coagulable nitrogen does not guarantee a mild grade of intoxication. We are convinced that a knowledge of the blood non-coagulable nitrogen is of considerable clinical value in the prognosis of acute abdominal conditions.

It should be kept in mind that the urea nitrogen as well as creatinine nitrogen may show high values in these conditions, and these points may be of value in differential diagnosis. It is established that other conditions besides chronic nephritis may show a marked increase in the creatinine and urea nitrogen of the blood. It should be recalled that "creatinine rises above 2.5 mg. per 100 cc. of blood almost without exception only in conditions with renal involvement" (Myers and Lough (6)). The conditions studied by us show a high creatinine fraction and constitute exceptions to this statement.

It will be noted that the undetermined nitrogen in these experiments is unusually high—more so than in cases of nephritis with high non-coagulable nitrogen. This may be a peculiarity of this type of intoxication as contrasted with simple retention of nitrogenous material, and a study of this point may bring out much valuable information.

Having established the fact that the non-coagulable nitrogen of the blood is much increased in many cases of intestinal obstruction or of closed intestinal loops, we may now ask: Why does not the kidney eliminate these substances immediately? The kidneys are normal in gross and by functional tests in practically all cases. There can be no true retention because of impaired kidney function. There may be two or more factors concerned. We know that in intestinal obstruction the current of fluid is mainly out of the body and by way of the intestinal tract, and it is possible that this favors the accumulation of certain products in the blood stream. The kidneys excrete small amounts of highly concentrated urine.

It is to be remembered, too, that injection of a small amount of a toxic proteose may cause a great rise in non-coagulable nitrogen in

the blood; for example, a rise from 40 to 80 mg. in 3 hours. This cannot be due to lack of elimination, and we must assume destruction of body protein to account for this remarkable change. We may assume that any acute proteose intoxication may be associated with a similar rapid rise in non-coagulable nitrogen in the blood. When we have more information about this point, we may better understand the manner in which the toxic proteoses injure the body and perhaps the various methods of body defense.

General peritonitis is often associated with a definite rise in non-coagulable nitrogen of the blood. How may we explain this observation? It may be argued that paralytic ileus is alone responsible, and this may be true in part. However, we think it important that a toxic proteose can be isolated from the exudate in cases of general peritonitis, and obviously must be absorbed by the host. The proteose intoxication may well be responsible for this change in non-coagulable nitrogen. We hope to report further on this point in the near future.

SUMMARY.

Intestinal obstruction, as a rule, is associated with an increasing amount of non-coagulable nitrogen in the blood. With acute intoxication the rise in non-coagulable nitrogen may be rapid and reach as high as three or even ten times normal. With more chronic intoxication there may be little or no rise in the blood non-coagulable nitrogen.

Closed intestinal loops show exactly the same picture, and, when combined with obstruction, may give very high nitrogen readings.

Acute proteose intoxication due to injection of a pure proteose will show a prompt rise in blood non-coagulable nitrogen, even an increase of 100 per cent within 3 or 4 hours.

These intoxications also show a high blood content of creatinine and urea. The residual or undetermined nitrogen may be very high.

A human case of intestinal obstruction with autopsy presents blood findings exactly similar to those observed in many animal experiments.

Clinically the non-coagulable nitrogen of the blood may give information of value in intestinal obstruction. A high reading means

a grave intoxication, but a low reading may be observed in some fatal cases and gives no assurance that a fatal intoxication may not supervene.

The kidneys in practically all these experiments are normal in all respects.

It is possible that protein or tissue destruction rather than impaired eliminative function is responsible for the rise in non-coagulable nitrogen of the blood in these acute intoxications.

Transfusions of dextrose solutions often benefit intestinal obstruction, and may depress the level of the non-coagulable nitrogen in the blood. Some cases show no change in non-coagulable nitrogen following transfusions and diuresis, and, as a rule, such cases present the most severe intoxication.

The writers wish to express their appreciation of the assistance given by Dr. A. R. Kilgore and Dr. T. Stearns of the University of California Hospital.

BIBLIOGRAPHY.

1. Tileston, W., and Comfort, C. W., Jr., *Arch. Int. Med.*, 1914, xiv, 620.
2. Folin, O., *Jour. Biol. Chem.*, 1914, xvii, 475.
3. Van Slyke, D. D., and Cullen, G. E., *Jour. Biol. Chem.*, 1914, xix, 211.
4. Benedict, S. R., *Jour. Biol. Chem.*, 1915, xx, 629.
5. Whipple, G. H., Rodenbaugh, F. H., and Kilgore, A. R., *Jour. Exper. Med.*, 1916, xxiii, 123.
6. Myers, V. C., and Lough, W. G., *Arch. Int. Med.*, 1915, xvi, 536.

THE ORIGIN AND STRUCTURE OF A FIBROUS TISSUE FORMED IN WOUND HEALING.

By GEORGE A. BAITSELL, Ph.D.

(*From the Osborn Zoological Laboratory of Yale University, New Haven.*)

Plates 106 to 109.

(Received for publication, March 14, 1916.)

INTRODUCTION.

In a previous paper[1] the author showed that in living cultures of adult frog tissues there occurs, in many instances, a transformation of the plasma clot in which the living tissue is imbedded. This transformation results in a consolidation or fusion of the elements of the fibrin net and a consequent formation from it of a fibrous tissue which is identical in its form and structure and in many of its staining reactions with a regular collagenous connective tissue. It was felt that, whether or not this new fibrous tissue directly formed from the fibrin clot remained as a permanent connective tissue, such a reaction must play a fundamental part in the processes attendant upon wound healing. The present paper[2] gives the results obtained from an extensive series of experiments undertaken for the purpose of studying the action and fate of the fibrin clot formed during wound healing, and they give evidence that in the healing of skin wounds in the frog a definite transformation of the fibrin clot takes place such as was found to occur in the tissue cultures. This transformation also results in the formation of a new fibrous tissue, without intracellular action, which is apparently identical with regular permanent connective tissue.

[1] Baitsell, G. A., The Origin and Structure of a Fibrous Tissue Which Appears in Living Cultures of Adult Frog Tissues, *Jour. Exper. Med.*, 1915, xxi, 455.

[2] A report of this work was given at the meeting of the American Association of Anatomists held at New Haven, Conn., Dec. 28–30, 1915. An abstract appears in the proceedings in the *Anat. Rec.*, 1916, x, 175.

Material and Methods.

It was thought best to carry on the experiments in wound healing with the frog inasmuch as the tissues used in the previous work with living tissue cultures had been obtained from this animal. During the present experiments thirty-one animals were used and a total of fifty operations were performed. The process of wound healing has been studied at various stages both in the living animals and in the preserved material. For the study of the preserved material pieces of the tissues containing the wounds were removed at various times after the operations. These were preserved in Zenker's solution, imbedded in paraffin, and sections, in various planes, were made at 10 μ. In general the stain used has been Mallory's connective tissue stain modified according to Mall.[3] Mallory's stain, unmodified, and the Van Gieson picro-fuchsin stain have also been used for comparison. A discussion of the staining reactions will be found in a later section of this paper.

In the preliminary experiments, the semimembranosus muscle lying on the dorsal surface of the hind legs of the frog was used. After anesthetization the animal was placed on an operating board dorsal surface up. The skin was then sterilized by washing it with a 0.001 per cent solution of mercuric chloride. This method of sterilization appears to be adequate for this work and there has been very little trouble from infection. An incision of about 5 to 7 mm. in length was then made in the median dorsal surface of either or both of the hind legs. The cut edges of the skin were separated and held apart and a cube of the underlying muscle tissue measuring about 2 mm. was removed. The cavity thus formed in the muscle tissue was either filled with plasma which had been obtained in the usual manner[4] from another frog, or by the blood and lymph which flowed into the wound from the cut edges. After the operation the frog was held in position for a few minutes until the clot had formed in the wound.

The results obtained from these experiments were not satisfactory. The many movements of the legs and the consequent expansion and

[3] Mall, F. P., On the Development of the Connective Tissues from the Connective-Tissue Syncytium, *Am. Jour. Anat.*, 1901–02, i, 338.

[4] Baitsell, G. A., The Origin and Structure of a Fibrous Tissue Which Appears in Living Cultures of Adult Frog Tissues, *Jour. Exper. Med.*, 1915, xxi, 456.

contraction of the muscle fibers resulted, in many cases, in a breaking up of the plasma clot in the wound cavity and its destruction, so that it was impossible to make a study of it. The friction of the overlying skin of the leg during these movements also tended to make the operations unsuitable for the present study of the fibrin clot. In a number of these experiments which were first made, stitches were taken in the skin to hold the cut edges together and in their proper positions. In the later operations it was found that this was not necessary for it was observed that when a cut was made in the skin, as noted above, if the animal remained quiet for a few minutes after the operation, the coagulation of the blood and lymph formed a tissue of sufficient strength to hold the cut edges of the skin in their proper position. It appeared evident from this that wounds in the frog skin would afford a suitable field for the study of the process of wound healing, particularly with regard to the action and fate of the fibrin clot. Later experiments showed that the skin lying on the posterior dorsal surface of the animal offered an even better field for these experiments than did the skin of the legs inasmuch as it is not subject to an almost continual, and at times violent strain as the result of leg movements. Wounds in both these regions have been studied and form the basis of the results given in the present paper.

Observations on the Process of Wound Healing in the Skin of Living Frogs.

The healing of various types of wounds has been observed in the living animals. In the first type of experiments, a simple incision was made in the skin of the frog either on the dorsal surface of the leg or on the back. As far as could be told by the observations on the living animal these wounds healed very quickly. The formation of the coagulation tissue as a result of the clotting of the blood plasma or lymph present in the wound occurred within a few minutes after the wound was made. As a result the cut edges were firmly held in position and the frog could then be placed in the aquarium without injury to the new tissue. The animals examined 24 hours later showed only slight evidence of the wound. The cut edges were still firmly held and all that could be noted was the light colored line of coagu-

lation tissue marking the extent of the incision. In the course of the next day or so the epithelial cells moved over this area and obliterated all evidences of the wound.

In the second type of experiments a piece of skin measuring about 5 by 2 mm. was detached on three sides. The fourth side (2 mm.) was left attached to the body. In this way a flap of skin was formed which was severed from the body except at one end. In such cases the coagulation tissue which quickly formed held all the cut edges of the skin and the loose flap in place, and thus an operation which one would naturally expect, without the aid of sutures to hold the edges in place, to have left a large open wound in the skin with a consequent slow healing was permanently closed within a few minutes after the operation. In the following days one could observe, as in the previous type of experiments, a gradual obliteration of the cuts by the epithelial cells which moved out over the coagulation tissue from the cut edges of the skin.

In the third type of experiments a piece of skin from the median posterior region of the back, measuring about 5 mm. square, was completely severed from the body of the animal, placed in sterile Ringer solution for a few minutes, and then replaced in the cavity from which it had previously been taken. The observations showed that no other support than the coagulation tissue which formed was necessary in order to hold such a piece of skin in position in the wound cavity, and the complete obliteration of the wound occurred in a few days by the movement of the epithelial cells over the coagulation tissue. The results of other experiments of this type showed that the living skin from another animal or a dead tissue, such as the stratum compactum obtained by pancreatin digestion of adult frog skin, could be transplanted into a wound cavity in the skin and retained there by the action of the coagulation tissue alone. In a later paper it is hoped that more complete results regarding such skin transplantation can be presented. The only fact regarding them which should be emphasized in the present paper is the action of the coagulation tissue in the wound cavities as shown by its ability to hold the transplant in position without the aid of sutures.

In the last type of experiments, a piece of skin, varying in size from 3 to 5 mm. square, was completely removed from the body. The resulting cavity was quickly filled with the coagulation tissue. In this

type of experiment it was found that in some cases, particularly if the cavity caused by the removal of a piece of skin was large, it was better to supply additional plasma obtained from another animal in order that the wound cavity might be completely filled. A wound of this type (Fig. 1) gives the best opportunity for the study of the coagulation tissue. It forms a hyaline mass in the wound cavity and is glossy and transparent, and until it has been covered by the epithelial cells which move in from the surrounding areas the underlying muscle tissue can be seen through it. This fibrin clot or coagulation tissue when it is first formed may sometimes be destroyed by extremely violent movements of the animal, especially if the wound be a large one. An increase in the strength of the new tissue soon becomes evident and in a few days it appears to be as immune from injury as regular skin tissue. As far as can be told by the observations on the living animals, this coagulation tissue formed by the clotting of the plasma in the wound remains permanently and takes the place of the piece of skin tissue which was removed. From this it is clear that in a skin wound in the frog, if the conditions are right, a new tissue can be formed almost immediately after the injury which is sufficiently strong to retain its place in the wound cavity and to hold the cut edges in position. The wound, to all appearances, is thus healed without waiting for a process of regeneration of tissues by cell division. Probably the most impressive part of this process of wound healing, to an observer, is the rapidity with which it takes place. The coagulation of the plasma closes the wound almost immediately and then, evidently by a transformation of this fibrin clot, a firm, resistant, and apparently permanent tissue is formed which serves as a regular connective tissue.

During the course of the experiments, observations have also been made on the healing of wounds in which no coagulation tissue was present. Such a condition may arise either because of the lack of a sufficient amount of plasma in the wound at the time it was made, because of a later destruction of the coagulation tissue as a result of tension caused by too vigorous movements, or because of an infection. In cases of this kind the process of wound healing in the skin of a frog is a comparatively long and slow one, and in some cases results in the death of the animal by a later infection received through the open wound.

Study of the Prepared Material.

In Fig. 1 a view of a wound in the skin of a frog is shown *in toto* at a magnification of about 15 diameters. This wound was removed from the animal and preserved when it was three days old. The wound, caused by the removal of a piece of skin, originally measured about 5 by 2 mm. The figure was drawn with the aid of a Zeiss binocular microscope from the under surface of the skin and shows the wound surrounded by the skin tissue and filled with the coagulation tissue (fibrin clot) which was formed within a few minutes after the wound was made by a clotting of the blood plasma and lymph. Several large blood vessels are shown, some of which run into the coagulation tissue. Some of these vessels appear to be enlargements of the vessels supplying the skin in that region, while others apparently arise in the underlying muscles. In some cases the wound is found to be attached to the muscles by a number of small vessels.

A transverse section of the same preparation is shown in Fig. 2 at a magnification of 77 diameters. At either end of this figure may be noted the cut ends of the skin which mark the boundaries of the wound. Connecting these can be seen the bridge of new tissue which has formed in the wound cavity. Lying above this coagulation tissue are several layers of epithelial cells which have wandered in from the skin tissue surrounding the wound. This movement of the epithelial cells begins very soon after the wound has been made and continues until, as shown in later stages (Fig. 6), they form a thick layer over the coagulation tissue. The epithelial cells in Fig. 2 are more numerous near the cut edges of the skin. At these points the coagulation tissue is drawn out into a rather thin strand which is attached to the cut edges of the skin and which serves to hold them in place. It can be noted in this figure that fibers are present in the coagulation tissue.

The observations on the living animals have shown that the coagulation tissue in a 3 day wound is very firm and resistant as compared with a young fibrin clot. From this it is evident that already some change has taken place in the clot and this fact can also be noted from the study of the prepared material. In Fig. 3 a portion of the coagulation tissue, from the same preparation as Fig. 2, is shown at

a magnification of 787 diameters. At this magnification one is able to see clearly the histological structure of the coagulation tissue. Quite large areas can be noted in this figure in which the fibrin net has not been changed but still retains its characteristic structure. In other regions, however, the fibrin net has been changed into a new fibrous tissue. In such areas it appears that there has been a fusion of the elements of the fibrin net to form long, wavy, fibrous bundles which are typical in structure and appearance to those found in various forms of connective tissues. This new fibrous tissue is apparently identical with that which forms in plasma clots in tissue cultures. The transformation of the fibrin clot in this wound into a new fibrous tissue has not been due to an intracellular action for, with the exception of some blood corpuscles which were present in the plasma when it clotted, some of which can be seen in Fig. 3, no cells of any kind are present in the new fibrous tissue except in the extreme edges where the new tissue is in contact with the cut edges of the skin. A thorough study of this point has been made in this and other similar preparations and it can be definitely stated that in wounds in the frog skin, as has previously been shown to be the case in tissue cultures, a direct transformation of a fibrin clot into a new fibrous tissue will occur without intracellular action.

In Fig. 4, which is a portion of a longitudinal section of a 4 day wound at a magnification of 787 diameters, a more complete transformation of the plasma clot is seen. The section from which this figure was drawn was taken from near the upper surface of the coagulation tissue; that is, from the dorsal portion of the clot lying just below the epithelial cells. At either edge of the coagulation tissue may be noted some of the epithelial cells which mark the boundary of the wound and which have grown down on both sides of the coagulation tissue. This downward growth of the epithelial cells can be noted in some of the transverse sections of the wound tissue (Fig. 2). A study of Fig. 4 shows that the fibrin net structure has disappeared and the new fibrous tissue has been formed which, as in Fig. 3, is composed of bands of fibrils showing the typical structure of regular connective tissue fibers. The new fibrous tissue is denser near the bottom of the figure and it is in this region that its structure is seen to the best advantage. All the coagulation tissue shown in

the figure has lost the original fibrin net structure, but in the less dense regions of this tissue, in which numerous vacuoles are present, the transformation into the new fibrous tissue has not been so complete. No fibroblast cells are present in this region of the clot and so in this case also the transformation of the fibrin clot into the new fibrous tissue has not been due to any intracellular action. In this preparation centrifuged blood plasma which had been obtained from another animal was placed in the wound to form the clot and this fact accounts for the almost complete absence of blood corpuscles. Those that are shown in this figure are in most cases matted together and are evidently undergoing degeneration.

In Fig. 5 is shown, at the same magnification as Fig. 4, another longitudinal section of the same preparation taken at a deeper level. In this figure a considerable number of both fibroblast cells and blood corpuscles are shown. In this figure a transformation of the fibrin clot has also occurred and a new fibrous tissue has been formed containing bundles of wavy fibers. These are particularly well shown to the left of the figure. In this region numerous cells are also present and as a result the bundles of fibers are more or less separated from each other. To the right of the figure fewer cells are present and the newly formed fibrous tissue is more closely massed together. The bundles of fibers, however, can be clearly seen.

A comparison of the new fibrous tissue shown in Figs. 3, 4, and 5, which has been formed through a transformation of the fibrin clot, with the fibrous tissue found in the plasma clot in living cultures of frog tissues, as noted in a previous paper,[5] shows that they are identical.

The conditions present in an older wound are shown in Fig. 6, which is a transverse section of a 12 to 13 day preparation at a magnification of 240 diameters. At either end of the figure can be seen the cut ends of the skin tissue which mark the boundaries of the wound and between these the new tissue which has been formed in the wound. Overlying this region are the epithelial cells which are piled up many layers thick. The wound cavity, except for a small portion

[5] Baitsell, G. A., The Origin and Structure of a Fibrous Tissue Which Appears in Living Cultures of Adult Frog Tissues, *Jour. Exper. Med.*, 1915, xxi, 478–79. Compare Fig. 3 of this paper with Figs. 11 and 17 of previous paper.

lying near the right end of the figure, is filled with the new fibrous tissue. Fibroblast cells are present all through the new tissue. In the region lying to the right a portion of the original fibrin clot apparently still remains. In Fig. 7, which was drawn from the same section as Fig. 6, this region of the wound is shown at a magnification of 787 diameters. The figure shows that the fibrin clot has lost its original structure, as shown for example in Fig. 3, and has become a more or less compact mass in which, at various places, it can be noted that fibers have formed. Around the edges of the fibrin mass, in particular, a great many of the bundles of fibers can be seen. These are attached to the fibrin clot and it is clear that they have arisen by a transformation of its elements and not from an intracellular action. The cells which are present stand out distinctly from the fibers, as shown in the figure, and show no connection with them.

In Fig. 8 is shown, at a magnification of 787 diameters, a longitudinal section of another wound which is practically the same age as the one shown in Figs. 6 and 7. In Fig. 8, however, we find that the formation of the new tissue has proceeded more rapidly and consequently the original fibrin net has been entirely transformed into the new fibrous tissue which fills the wound cavity. Blood vessels are forming in various regions in this new tissue and numerous cells, which for the most part show the typical spindle shape of fibroblast cells, are present but stand out clearly and distinctly from the fibers. The question of the relations existing between the new fibrous tissue and the cells which wander into it from the surrounding edges of the skin is one which has been studied with care. It has already been pointed out that the transformation of the fibrin clot occurs previous to the appearance of any cells, so that it cannot be due to an intracellular action (Figs. 3 and 4). A study of the preparations shows that the cells when they first appear in the new fibrous tissue are round in shape (Figs. 5 and 7). Later they elongate and assume the typical spindle shape of fibroblast cells (Fig. 8). This change in the shape is apparently due to the stereotropic action of the cells in moving along the fibers.[6] The movement of the cells in the newly formed fibrous

[6] Harrison, R. G., The Reaction of Embryonic Cells to Solid Structures, *Jour. Exper. Zool.*, 1914, xvii, 521.

tissue appears to result in a separation of the bundles of fibers and we have as a result what, to all appearances, is a typical scar tissue. With the Mallory stain the cells are colored yellow and they are distinctly differentiated from the fibers of the new tissue which are colored blue by the stain. The preparations do not show any evidence of a digestion by the cells of the new fibrous tissue or of any attempt by them to form new fibers intracellularly. The condition shown in Fig. 8 is typical of the condition found in much older wounds (3 to 4 weeks) and the evidence appears clear from these results that, in the healing of skin wounds in the frog, the new fibrous tissue present in the wound which has been formed by a direct transformation of the fibrin clot remains as the permanent connective tissue.

Nature of the New Fibrous Tissue.

The appearance, structure, and staining reaction with Mallory's stain of the new fibrous tissue are, as shown in the previous paper, identical with those of regularly formed connective tissues. In an endeavor to settle definitely the real relationship existing between regular frog connective tissue and the new fibrous tissue several series of tests have been made.

Staining Reactions of the Fibrous Tissue Formed in Wounds.—The Mallory connective tissue stain, either modified or unmodified, has proved to be more specific in its reaction than any other stain that has thus far been used in the work, although various other methods have been tried. This stain will color connective tissue fibers of the frog an almost perfect ultramarine blue if they are not too closely packed together. If, for example, a piece of skin from an adult frog is stained with Mallory's stain it will be found, in general, that the stratum spongiosum, in which the bundles of connective tissue fibers are loosely packed together and in which the individual fibrils can be distinguished, will be colored an ultramarine blue. On the other hand, in the stratum compactum of the same preparation in which the bundles of fibers are closely massed, it will be found that this layer will be stained a color varying from yellow to bright red, depending upon the manipulation of the stain.

The new fibrous tissue formed in the clots shows the same color re-

action to a Mallory stain as does the connective tissue in the skin. When the bundles of fibers in the new fibrous tissue are not closely matted together, as shown for example in Figs. 3, 4, and 5, the Mallory stain gives them a typical blue color as in the stratum spongiosum of the skin. On the other hand, when the new fibrous tissue is very compact and the individual fibrils composing the bundles cannot be distinguished, then, in many cases, the result will be a color varying from yellow to red, similar to that found in the stratum compactum of the skin. The same color also generally results from the Mallory stain in very young preparations in which the clot has not been changed into the new fibrous tissue but retains its original structure.

With a Van Gieson picro-fuchsin stain the new fibrous tissue shows a negative reaction. This stain, however, does not appear to be a specific one for connective tissues of the frog. The results show that it acts in just the opposite way that Mallory's stain does, in that it only gives the characteristic red color for connective tissues when a heavy tissue is present in which the bundles of fibers are closely massed together. In the frog skin, for example, the stratum compactum occasionally stains red, whereas in the stratum spongiosum an entirely negative reaction is obtained. In most cases, however, all the connective tissues of the frog skin as well as those from other parts of the body give a negative color reaction with this stain. In this connection a series of staining tests were made with embryonic connective tissue from the tail of a 50 mm. tadpole. It consists of a mass of connective tissue fibers loosely packed together. These fibers will take a typical stain with Mallory's stain, but with Van Gieson's the reaction is negative, as it is with the new fibrous tissue. The results in brief from all these experiments show that the Van Gieson's stain is not specific for connective tissue fibers in the frog whether embryonic or adult, and the same is true for its reaction with the new fibrous tissue.

Digestion Tests of the Fibrous Tissue Formed in Wounds.—Of all the tests used to distinguish between fibrin and connective tissues, the digestion tests are regarded as being the most specific and conclusive. A young fibrin clot is easily digested in a pancreatin solution whereas connective tissues resist the action. In the previous paper it was noted that the transformed fibrin net in the tissue cultures was easily dissolved in

pancreatin, and this fact gave evidence that the new fibrous tissue was fibrin in character although greatly changed in appearance from the typical fibrin net. However, it was also pointed out that in this case the test was not conclusive, inasmuch as the newly formed fibers in the clot were imbedded in a fibrin net which was not completely transformed, and the digestion of the unchanged fibrin clot by the pancreatin would naturally cause a breaking down and scattering of the fibers which had formed in it, and remained connected with it. In other words, the surrounding unchanged fibrin constituted the sole support of the fibers which had formed from a part of it.[7] This close relation existing between the fibrin clot and the new fibrous tissue also prevents the digestion tests being used in the present experiments to furnish a conclusive answer as to the real nature of the transformed fibrin net tissue in the clots formed in wound healing.

In this connection the results secured by a series of experiments in which the embryonic connective tissues of the tadpole were subjected to pancreatin digestion should be noted. In these experiments pieces of skin, taken from tadpoles ranging in length from 7 mm. to large tadpoles of about 70 mm. just before metamorphosis, were placed in pancreatin solutions of uniform strength[8] and digested over night at 38°C. In all these experiments a complete digestion of the tadpole skin occurred. This same result was obtained when the connective tissue from the tails of tadpoles of various ages was digested in pancreatin solutions. These young fibers, as has been noted, stain typically with Mallory's stain and are apparently fully formed. Nevertheless they can be completely digested in pancreatin in a few hours. These results with embryonic connective tissues are entirely different from those obtained when connective tissues from an adult frog are subjected to the same treatment, for the experiments show that the mature connective tissues in the skin of an adult frog are able to resist the action of the pancreatin and remain as a mass of tissue which, as noted above, could be used in the implantation experiments.

[7] Baitsell, G. A., The Origin and Structure of a Fibrous Tissue Which Appears in Living Cultures of Adult Frog Tissues, *Jour. Exper. Med.*, 1915, xxi, 473.

[8] The pancreatin extract used in all these digestion experiments was obtained by the well known Roberts' method. The author is indebted to Professor F. P. Underhill for his assistance in this matter.

These results demonstrate that the reaction of the embryonic connective tissues of the frog to pancreatin digestion is the opposite of that of adult connective tissue.

The results of both the staining and digestion tests, in brief, show that with these methods one is unable to differentiate between the new fibrous tissue, formed by a transformation of the fibrin clots, and embryonic connective tissues of the tadpole, and that the connective tissues of the adult, while reacting in the same way to the stains, can be differentiated from both the new fibrous tissue and embryonic connective tissue by their ability to resist the pancreatin digestion.

There is the possibility that the same resistance to pancreatin digestion which must develop in embryonic connective tissue as it becomes mature, as shown by the digestion tests on the mature and embryonic connective tissues, might also develop in time in the new fibrous tissue which has resulted through a transformation of the fibrin clot, for it is well known that old fibrin clots become highly resistant to various agents which will entirely dissolve younger clots. Mall[9] says: "Fresh fibrin is easily dissolved in dilute acid or digested in pancreatin. After fibrin becomes older, as in fibrinous deposits, the fibrils become very resistant and are not dissolved in boiling acetic acid, 20 per cent, in cold concentrated KOH, H_2SO_4, HCl, HNO_3, or nitro-hydrochloric acid." What changes take place in these fibrinous deposits that cause such an increase in their resisting powers is apparently not known. From the present results one would naturally suggest that in such cases, in the course of time, a complete transformation of the fibrin clot into the new fibrous tissue had occurred and that this transformed tissue, when old enough, was able to resist the action of pancreatin and various other destructive agents.

This point has proven to be one which is difficult of solution. The fact that even the fully formed connective tissue of large tadpoles is unable to resist the pancreatin digestion makes it impossible to expect that one could ever keep tissue cultures alive long enough to secure a new fibrous tissue from the fibrin clot which would be able to

[9] Mall, F. P., Reticulated Tissue, and Its Relation to the Connective Tissue Fibrils, *Johns Hopkins Hosp. Rep.*, 1896, i, 182, 194.

resist the pancreatin digestion and thus show definitely that it could not be distinguished from the regular connective tissues in this regard. In wounds the problem is complicated by the presence of the cells which wander into them and through the new fibrous tissue soon after it has been formed from the fibrin clot. The preparations, as stated above, do not show any evidence of an attempt by these cells to form new fibers intracellularly, but, nevertheless, their presence in the wound for the long period of time which evidently is necessary for the new fibrous tissue to become resistant, makes the problem a difficult one.

DISCUSSION.

The experiments reported in the present paper demonstrate that in wounds made in the skin of adult frogs there occurs, as has previously been shown to be the case in living cultures of adult frog tissues, a direct transformation of the fibrin clot into a new fibrous tissue without any intracellular action. This newly formed fibrous tissue which fills the wound space is apparently identical in appearance, structure, function, and staining reactions with regularly formed permanent connective tissue. It differs from adult connective tissue in the skin of the frog in its reaction with pancreatin digestion. However, this test, as well as all others that have so far been tried, fails to differentiate between the new fibrous tissue and young connective tissue found in tadpoles of various stages.

Two views might be held with reference to the fate of this new fibrous tissue directly formed from the fibrin clot. The first is that it is only a temporary tissue and that it will be replaced in time by a permanent connective tissue formed through an intracellular action of the fibroblasts. This is in accord with the generally prevailing views[10] held at present, which are that the fibrin, in wounds healing by first intention, forms a temporary tissue which holds the wound surfaces in place and stops up the wound thus preventing infection. Later the fibrin disappears, not by liquefaction, but through an actual consumption by the new tissue cells which show a positive chemotropism to the fibrin mass and are thus attracted into the fibrin clot

[10] For a general discussion and summary see Marchand, F., Der Process der Wundheilung, Stuttgart, 1901, 52–55.

in large numbers. The permanent tissues which go to fill the wound space are then formed by these cells through an intracellular action.

The other view is that this new fibrous tissue remains in the wound as a permanent connective tissue. That is to say, at least in wound healing in the frog skin, a reaction occurs which is able to transform a fibrin clot by a direct intercellular action into a permanent fibrous connective tissue. This view is supported by the work of Hertzler[11] who finds that, in experimental peritoneal adhesions in the dog, a new fibrous tissue, which he believes remains permanently, is formed by a transformation of a fibrin net. Speaking of the formation of this fibrous tissue, he says:[12] "My researches have convinced me that the cell is not primarily the active agent, but that the initial processes are chemical and are identical with those of blood coagulation, the cell playing an entirely secondary rôle."

The results obtained in the present experiments also give evidence in favor of the latter view. The fibroblast cells which wander into the new fibrous tissue apparently do not digest the fibers which have previously been formed, nor is there any evidence revealed, by a thorough study of the preparations, of an attempt by these cells to form new fibers intracellularly. Their only apparent action is to break up the larger bundles of fibers by their movements among them. In a wound of about 12 days a typical scar tissue is generally present (Fig. 8), and later stages (3 to 4 weeks) do not show any further changes in the structure of the connective tissue present in the wound.

SUMMARY.

1. In experimental wounds, made by removing various sized pieces of skin from the frog, there is a rapid coagulation of the blood plasma and lymph to form a coagulation tissue which fills the wound cavity.

2. The observations on the living animals show that the coagulation

[11] Hertzler, A. E., Pseudoperitoneum, Varicosity of the Peritoneum and Sclerosis of the Mesentery. With a Preliminary Note on the Development of Fibrous Tissue, *Jour. Am. Med. Assn.*, 1910, liv, 351; The Development of Fibrous Tissues in Peritoneal Adhesions, *Anat. Rec.*, 1915, ix, 83.

[12] Hertzler, A. E., Pseudoperitoneum, Varicosity of the Peritoneum and Sclerosis of the Mesentery. With a Preliminary Note on the Development of Fibrous Tissue, *Jour. Am. Med. Assn.*, 1910, liv, 352.

tissue becomes more and more resistant and is generally of sufficient strength to hold the cut edges of the wound in place and to retain its position in the wound cavity. It serves, at least temporarily, as a connective tissue and as a base for the epithelial cells which rapidly move in from all the cut edges and cover the wound.

3. The study of the prepared sections of wound tissue show that at first in the coagulation tissue, formed as a result of the clotting of blood and lymph, a typical fibrin net is present in the wound (Fig. 3). Later this fibrin net is transformed into a new fibrous tissue containing bundles of wavy fibers in which, in many instances, the individual fibrils can be noted (Fig. 4). This transformation of the clot and the formation of the new fibrous tissue takes place before the tissue cells wander into the coagulation tissue and therefore cannot be due to an intracellular action. It is a direct transformation of the fibrin clot and is identical with the process which was previously found to take place in the fibrin clots in living cultures of adult frog tissues.

4. The tissue cells, which later move into the new fibrous tissue in large numbers from the surrounding areas, do not digest the fibers but, apparently by their movements, cause a division of the large bundles into smaller ones (Fig. 5). These cells when they first appear in the fibrous tissue are rounded, but later they assume the typical elongated spindle shape of fibroblast cells (Fig. 8). The preparations do not show any connection between these spindle-shaped cells and the fibers which had already formed, nor is there any evidence of a later attempt by them to form new fibers intracellularly.

5. The staining reactions of the new fibrous tissue appear to be identical with the staining reactions of the connective tissue in frog skin. However, the new tissue can be digested in pancreatin and in this reaction it differs from the connective tissue in the skin of the adult frog. On the other hand, extensive experiments with pancreatin on embryonic but fully formed connective tissue, obtained from the tail and skin of tadpoles of various ages, show that pancreatin will digest it just as it does the newly formed fibrous tissue.

EXPLANATION OF PLATES.

All the figures, with the exception of Fig. 1, were drawn from prepared material which had been preserved in Zenker's solution, sectioned at 10 μ, and stained with Mallory's connective tissue stain.

Plate 106.

Fig. 1. 3 day wound from back of frog, shown *in toto* from the under surface. × 15. A piece of skin measuring about 5 by 2 mm. was removed and the cavity thus formed is shown filled with the coagulation tissue (c.t.). A considerable area of the skin tissue (sk.t.) surrounding the wound is shown, and blood vessels (b.v.) are also present.

Fig. 2. Transverse section of the preparation shown in Fig. 1. × 77. Cut edges of the skin tissue (sk.t.) marking boundaries of the wound are shown at both ends of the figure. These are connected by the coagulation tissue (c.t.) in which fibers (fib.) can be noted at various places. Epithelial cells (ep.c.) which have moved in from the surrounding areas overlie the coagulation tissue. Section of blood vessel (b.v.) and numerous blood corpuscles (b.c.) are also shown.

Plate 107.

Fig. 3. Transverse section showing a portion of the coagulation tissue from the same preparation as Figs. 1 and 2. × 787. The figure shows the structure of the coagulation tissue present in a 3 day wound. Some of this tissue retains the fibrin net (f.n.) structure of the original fibrin clot. Other areas show new fibrous tissue (n.f.t.) which has been formed by a transformation of the elements of the fibrin net. Numerous blood corpuscles (b.c.) are present, but no fibroblast cells.

Fig. 4. Longitudinal section of a 4 day wound in the leg of a frog. × 787. The coagulation tissue has been transformed into the new fibrous tissue (n.f.t.) in which bundles of wavy fibers can be seen. Transformation is most complete in the denser region of coagulation tissue lying to the right of the figure. No fibroblast cells are present, but a few blood corpuscles (b.c.) are shown which are evidently undergoing degeneration.

Fig. 5. Longitudinal section from the preparation shown in Fig. 4. × 787. The section from which this drawing was made was taken at a deeper level of the wound than Fig. 4; *i.e.*, just above the underlying muscle tissue. To the right the new fibrous tissue (n.f.t.) is more dense, but even here the fibrous structure can be seen. To the left it is broken up into wavy fibrous bundles among which numerous cells (fbl.c.) are appearing. Blood corpuscles (b.c.) are also present.

Plate 108.

Fig. 6. Transverse section of a 12 to 13 day wound preparation. × 240. Cut ends of the skin tissue (sk.t.) are shown at the right and left of the figure.

The wound cavity is almost filled with the new fibrous tissue (N.F.T.) except for a small portion of fibrin clot (F.C.) lying to the right. Epithelial cells (EP.C.) are piled up many layers thick above the new tissue. Cells (FBL.C.) are also present in the new tissue. Compare Fig. 7.

Fig. 7. A portion of the preparation shown in Fig. 6. × 787. This drawing shows the remains of a fibrin clot (F.C.) lying to the right in Fig. 6. It has lost its original fibrin net structure and has become a more or less compact mass. Numerous fibers can be seen running through it, and around the edges are a great many fibers which have resulted from a transformation of the fibrin clot and which go to form the new fibrous tissue (N.F.T.). Numerous rounded cells (FBL.C.) are present, some of which are beginning to assume the typical spindle shape of fibroblast cells. They stand out clearly and show no connection with the fibers.

Plate 109.

Fig. 8. Longitudinal section of another 12 to 13 day wound preparation. × 787. This figure shows complete transformation of coagulation tissue into new fibrous tissue (N.F.T.) which fills the wound cavity between the cut edges of the skin tissue (SK.T.). Blood vessels (B.V.) are forming and numerous spindle-shaped fibroblast cells (FBL.C.) are present. These cells stretch out along the fibers which had previously been formed through a transformation of the fibrin net and apparently separate the bundles. They do not digest the fibers nor is there any evidence of an attempt by them to form new fibers intracellularly.

THE ABSORPTION OF ADRENALIN AFTER INTRATRACHEAL INJECTION.*

By J. AUER, M.D., and FREDERICK L. GATES, M.D.

(*From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 11, 1916.)

In order to subject a living organism to the systemic action of any soluble substance, it is obvious that the substance must first reach the circulating fluids of this organism; from the lymph and blood streams the drug then may pass into the tissues and exert its effect. The main routes available for bringing any substance into contact with the tissues are as follows: (1) by introduction into the gastro-intestinal canal; (2) by subcutaneous, intramuscular, intravenous, or intraspinal injection; (3) by inunction through the skin; and (4) through the respiratory tract.

Of these routes the first two are most frequently employed in human therapeutics and in the laboratory. Inunction is used only exceptionally at the present time, and practically the only drug administered through the skin for its systemic action is mercury.

The respiratory route also is not utilized to any extent, except for purely local effects, when it is desired to incorporate a drug. Syphilis was occasionally treated by allowing the patient to inhale the sublimated metal, but this method was never extensively employed because of the impossibility of judging the dose. Local affections of the respiratory passages, for example, laryngitis, bronchitis, and bronchiectasis, are treated by allowing the patient to inhale the vapors of boiling water to which various substances (creosote, eucalyptol, opium preparations, etc.) have been added. Experimentally the respiratory passages are practically not utilized when a drug is to be administered for its general action.

*A preliminary report was published in the Proceedings of the Pharmacological Society (*Jour. Pharm. and Exper. Therap.*, 1914–15, vi, 608).

Among the relatively few experimenters who have used the intratracheal method for this purpose we may mention Külbs,[1] who injected rabbits repeatedly for a number of days with adrenalin, introducing the hypodermic needle into the trachea through the skin of the neck. He observed cough after the injection, and twice an animal died a few minutes after the injection, from pulmonary edema. In those animals which survived the injections, which were given every day or every other day for 22 to 78 days, the total amount being 3.4 to 14 cc. of adrenalin, he found the same macroscopic and microscopic alterations, though of smaller extent, which he obtained after intravenous injection of adrenalin.

Ephraim[2] in a series of papers reports the results of endobronchial treatment of chronic bronchitis and asthma in the human subject. Various drugs, including adrenalin, were introduced as a fine spray into the bronchi, often through a bronchoscope. The effects obtained were chiefly local; even after the bronchial administration of 1 mg. of adrenalin by means of a nebulizer, he obtained no definite rise of blood pressure. In a dog, however, the same method yielded a powerful rise of blood pressure when a large amount, 2 mg., was used.

The failure of Ephraim to obtain a rise of pressure after the administration of 1 mg. of adrenalin endobronchially in the human subject is perhaps attributable to the method. Ephraim himself observed in rabbits that the spray of a colored solution from a nebulizer, even when introduced into the trachea, did not reach the bronchi, the vapor being precipitated near the point of application. In the same way the endobronchial spray of adrenalin was possibly precipitated in the larger bronchi so that only negligible amounts reached the alveolar region where, as we shall show, the absorption is best.

That even colloids can be absorbed with rapidity when injected intratracheally is shown by Ishioka.[3] Ishioka sensitized guinea pigs by the subcutaneous injection of human serum and after 12 to 15 days injected 0.05 to 0.1 cc. of human serum into the trachea of these animals with the object of producing an anaphylactic pneumonia. He observed the milder anaphylactic symptoms in the majority of his experiments, but in two instances acute death with the typical lung picture resulted.

The absorptive capacity of the supratracheal respiratory passages does not concern us here; references concerning the effect of inhaled substances will be found in the papers by Ephraim.

Our own experiments will show that the intratracheal injections of adrenalin are rapidly absorbed even under disadvantageous conditions and exert a systemic effect, and also that this method may be of value therapeutically when a rapid action on the heart is desired.

[1] Külbs, *Arch. f. exper. Path. u. Pharm.*, 1905, liii, 150.

[2] Ephraim, A., *Deutsch. med. Wchnschr.*, 1911, xxxvii, 2079; 1912, xxxviii, 1453.

[3] Ishioka, S., *Deutsch. Arch. f. klin. Med.*, 1912, cvii, 500.

Methods.

The research was carried out entirely with rabbits, and the chief test substance employed was adrenalin. Adrenalin was chosen because its absorption could be readily detected by recording the blood pressure, and the character and degree of the resultant rise would give some indication of the speed and amount of absorption.

The animals were anesthetized by ether for the operative interferences. These were the insertion of a cannula into the right carotid artery; transection of the trachea with introduction and ligation of a wide glass tube about 2 cm. long into the distal stump so that the respiratory path was free and injection into the trachea easy; in some rabbits the tracheal cannula was inserted as near the thorax as possible and the lower end of the proximal trachea then ligated, thus converting the cervical trachea into a sac from which the absorption of adrenalin could be tested; in a number of experiments both vagi were sectioned in the neck.

The blood pressure was written by a mercury manometer connected with the artery by tubing filled with a half saturated solution of sodium sulphate.

The adrenalin used was generally the commercial solution in 0.1 per cent strength, preserved with chloretone. In some experiments, however, a solution was made from the commercial powder preparation, and this was used with or without the addition of chloretone.

The dose injected varied from 0.15 to 0.03 cc. per kilo of body weight. The maximum dose quoted was occasionally greatly exceeded when adrenalin was injected intramuscularly.

The solutions were injected into the respiratory passages in several ways. Usually the required amount was injected in 4 to 6 seconds from a Record tuberculin syringe into the tracheal cannula. In a number of experiments a filiform catheter was introduced into a bronchus and the solution then driven in by a gentle blast of air. A few times the solution was injected directly into the lung tissue by passing the hypodermic needle through the walls of the chest.

In a majority of the experiments doses of adrenalin were injected repeatedly, not only into the trachea but also into the erector spinæ muscles of the back.

The rabbits were always placed on an electric warming pad to reduce or prevent the loss of body heat.

EXPERIMENTAL RESULTS.

Twenty-three experiments were carried out in the adrenalin series; in ten of these tests both vagi were cut previous to the injection of the drug. The course of the experiments and the characteristic effects will be illustrated by a few typical protocols arranged in tabular form.

All rabbits tabulated were tracheotomized under light ether anesthesia; there was no insufflation of air except in No. 3 (Table I); vagi

were intact, except in No. 8 (Table IV); all injections into the trachea were made with a syringe through the tracheal tube.

TABLE I.

No. of animal.	Color. Sex. Weight.	No. of injection.	Method and site of injection.	Amount of adrenalin per kilo.	Latent period of blood pressure rise.	Blood pressure rise.	Duration of blood pressure rise.	Interval between injections.
				cc.	*sec.*	*mm.*	*min.*	*min.*
3		1	Catheter into bronchus.	0.23	12	64 (104–168)	8	
		2	Trachea.	0.23	—	45 (90–135)	9	9
	Gray ♀	3	"	0.23	6	25 (107–132)	9	10
Vagi intact.	2,210 gm.	4	Erector spinæ muscle.	0.23	No rise in 4 min.			10
		5	Erector spinæ muscle.	0.46	" " " 4 "			4
		6	Trachea.	0.23	7	24 (96–120)	18	4

Killed by medullary puncture after experiment. Lungs showed only a moderate degree of pulmonary edema.

6		1	Tracheal sac.	0.25	52	16 (91–107)	15	
		2	Trachea.	0.25	12	60 (100–160)	—	17
	Gray ♂	3	Tracheal sac.	0.25	No rise in 6 min.			16
Vagi intact.	2,100 gm.	4	Trachea.	0.25	3	40 (62–102)	10	8
		5	Erector spinæ muscle.	0.5	No rise in 7 min.			10
		6	Trachea.	0.25	4	26 (80–106)	7	10

Killed later by medullary puncture. The lower lobes of both lungs showed well marked pulmonary edema; slight in upper lobes.

TABLE II.

No. of animal.	Color. Sex. Weight.	No. of injection.	Method and site of injection.	Amount of adrenalin per kilo.	Latent period of blood pressure rise.	Blood pressure rise.	Duration of blood pressure rise.	Interval between injections.
				cc.	*sec.*	*mm.*	*min.*	*min.*
7		1	Trachea.	0.12	2	50 (100–150)	6	
		2	"	0.12	4	39 (88–127)	12	9
	Gray ♂	3	Erector spinæ muscle (left).	0.6	3	43 (99–142)	20	13
	1,610 gm.	4	Trachea.	0.12	8	21 (98–119)	10	21
Vagi intact.		5	"	0.12	5	25 (95–120)	12	15
		6	Erector spinæ muscle (right).	0.28	No rise in	8 min.		14
		7	Trachea.	0.12	3	24 (92–116)	21	8
		8	"	0.24	10	10 (102–112)	5	20

Killed later by medullary puncture. Lungs collapsed well and showed only a moderate degree of pulmonary edema.

8		1	Trachea.	0.21	6	64 (114–178)	15	
Vagi cut.	White ♀ 2,365 gm.	2	"	0.21	10	30 (110–140)	6	18
		3	"	0.21	8	16 (116–132)	9	8
		4	Erector spinæ muscle.	0.42	No rise in	4 min.		15

Pink fluid poured from trachea 13 min. after first dose. About 5 min. after last injection, blood pressure fell abruptly, convulsions, death. Lungs showed marked pulmonary edema.

TABLE III.

No. of animal.	Color. Sex. Weight.	No. of injection.	Method and site of injection.	Amount of adrenalin per kilo.	Latent period of blood pressure rise.	Blood pressure rise.	Duration of blood pressure rise.	Interval between injections.
				cc.	*sec.*	*mm.*	*min.*	*min.*
4		1	Erector spinæ muscle (left).	0.23	14	34 (106–140)	4	
		2	Jugular vein.	0.23	At once.	43 (107–150)	10	9
Vagi intact.	Gray 2,160 gm.	3	Trachea.	0.23	8	20 (110–130)	10	9
		4	Erector spinæ muscle (right).	0.23	No rise in 2 min.			11
		5	Trachea.	0.23	10	10 (100–110)	3	2
		6	Erector spinæ muscles.	0.23	No rise.			

After last injection foam and pink fluid poured from trachea (marked pulmonary edema).

A study of these typical protocols as given in Tables I, II, and III shows with clearness all the points we wish to emphasize, and they will now be considered in detail.

Latent Period.—The latent period elapsing between injection and the onset of the blood pressure rise varied between 2 and 38 seconds, but usually was less than 10 seconds; the general average of all first injections was 13 seconds. Repeated injections exerted no uniform effect on the length of the latent period. Thus in Experiment 7 the first dose of 0.12 cc. of adrenalin per kilo injected into the trachea surely reached a physiologically effective concentration in the blood within 2 seconds, for after that interval the blood pressure began to rise. The seventh injection of the same dose in the same place, 80 minutes later, exerted a blood pressure effect after a latent period of only 3 seconds, in spite of the fact that the animal had received intratracheally and intramuscularly in the interval between the two injections mentioned 1.24 cc. of adrenalin per kilo, divided into five doses. A still more striking example of the speed of absorption is

given in Experiment 8. In this experiment the vagi had been cut before administering the adrenalin, and the first dose of 0.21 cc. per kilo into the trachea caused a pulmonary edema of such an extent that foam and pinkish fluid poured from the tracheal cannula. Nevertheless, a repetition of the same dose in the same place a few minutes later exerted a good blood pressure effect after a latent period of only 10 seconds. In this instance the area available for absorption was undoubtedly reduced considerably by the pulmonary edema, yet the speed of absorption was still rapid. All the tabulated protocols show a similar rapid absorption after intratracheal injections.

The latent period or speed of absorption was not apparently affected by the amount of adrenalin injected; for example, in Experiment 3 the intrabronchial injection of 0.23 cc. per kilo by means of a catheter showed a latent period of 12 seconds before the blood pressure began to rise. In Experiment 7, on the other hand, a much smaller dose per kilo, 0.12 cc., injected into the trachea gave a blood pressure effect after a latent period of only 2 seconds.

Quantitative Absorption.—The quantitative absorption cannot be definitely established, yet the blood pressure rises obtained after the various injections furnish some indication of the amount of adrenalin absorbed. Examination of the absolute values of the blood pressure rises in mm. of mercury obtained after successive injections of the same dose of adrenalin in the same place, the lung passages for example, shows that the blood pressure effect in general decreases with the number of injections. Thus in Experiment 7 five doses of 0.12 cc. of adrenalin per kilo were injected into the trachea, the time intervals varying between 9 and 34 minutes if the interpolated intramuscular injections are included. The blood pressure rises after these five tracheal injections were 50, 39, 21, 25, and 24 mm. In Experiment 8 the successive decrease of effect is more pronounced. In this experiment 0.21 cc. of adrenalin per kilo into the trachea at intervals of 8 to 18 minutes gave the following rises of blood pressure: 64 ,30, and 16 mm. In this animal, however, the vagi had been sectioned previous to the first injection of adrenalin, and after the first dose a marked pulmonary edema developed during which fluid poured from the tracheal cannula. The presence of so much fluid decreased the absorptive area available, and in addition diluted the adrenalin with a colloidal

solution, rendering absorption still slower, yet in spite of this the speed of absorption was not appreciably delayed, the latent period being 10 and 8 seconds, but the amount absorbed, as judged by the pressure rises, showed practically 50 per cent decreases.

This decrease in effectiveness in causing a blood pressure rise shown by successive tracheal injections is in accord with the observations of Meltzer and Auer[4] that adrenalin diminishes absorption from the tissues. These authors demonstrated, among other facts, that the intravenous injection of adrenalin delays the absorption of strychnine or fluorescein from the subcutaneous tissue, and also that repeated intramuscular injections give a decreasing blood pressure effect, due to diminished absorption.

It will be noticed on examining the tables that all injections of adrenalin into the trachea gave some blood pressure effect; in other words, that some absorption took place even under unfavorable circumstances. A comparison was therefore made between the absorptive capacity of the lung and that of the erector spinæ muscles. The erector spinæ muscle was chosen because this thick mass of muscle is composed of fine fibers not separated into coarse fasciculi, like the glutei for example, and in addition is surrounded by a dense fascia which exerts pressure upon the injected substance, thus facilitating absorption. Injection into this muscle has been shown by Meltzer and Auer[4] to be practically equivalent to an intravenous injection.

The experimental test gave important results. It clearly appeared that the injection of several doses of adrenalin into the lung passages reduced the absorption from the erector spinæ muscles to such a degree that even double the intratracheal dose given intramuscularly did not enter the circulation in sufficient amount to affect the blood pressure; nevertheless, another injection of the original dose of adrenalin into the lungs promptly entered the circulation in sufficient concentration and amount to cause a blood pressure rise (see Experiments 3, 6, 7, and 4). This fact appears with especial clearness in Experiment 3. In this test the rabbit received three doses each of

[4] Meltzer, S. J., and Auer, J., *Tr. Assn. Am. Phys.*, 1904, xix, 207; *Jour. Exper. Med.*, 1905, vii, 59. Auer and Meltzer, *ibid.*, 1911, xiii, 328.

0.23 cc. of adrenalin per kilo into the lung, the time consumed being about 29 minutes. Each injection gave a rise of blood pressure (64, 45, and 25 mm.). Then the same dose was injected intramuscularly, but no blood pressure rise occurred in 4 minutes. The dose was then doubled and 0.46 cc. per kilo was injected into the other erector spinæ muscle, but again no blood pressure rise followed. 4 minutes later the original dose of 0.23 cc. was injected into the lung passages and after a latent period of 7 seconds the blood pressure began to rise and reached an absolute value of 24 mm. of mercury.

The failure to obtain a blood pressure effect from an intramuscular injection of adrenalin under the conditions mentioned is, however, not absolute. If the dose administered intramuscularly is increased sufficiently, enough adrenalin will be absorbed to cause a blood pressure rise. This is illustrated in Experiment 7. Two lung injections of 0.12 cc. of adrenalin each had been injected in about 22 minutes. Then five times the dose (0.6 cc. per kilo) was injected intramuscularly; after a latent period of 3 seconds the blood pressure rose 43 mm. That there was, nevertheless, a definitely diminished absorption from this large intramuscular injection is shown by the fact that the rise of blood pressure, 43 mm., is even less than that caused by the first lung injection of only one-fifth the dose, which latter raised the pressure 50 mm. Subsequent injections of adrenalin into the lungs all gave rises of blood pressure, but an interpolated intramuscular injection of double the pulmonary dose gave no blood pressure effect. The amount absorbed from the muscles had fallen below the level of a physiologically effective dose.

In some experiments the conditions were still further varied by preceding the lung injections of adrenalin by intramuscular and intravenous injections of the same substance. Experiment 4 is one of this type. The adrenalin dose was always 0.23 cc. per kilo. After an intramuscular and an intravenous injection, absorption of adrenalin from the lung was by no means prevented, the blood pressure rising 20 mm. after a latent period of 8 seconds. A subsequent intramuscular dose, however, produced no blood pressure rise within 2 minutes. Another lung injection even now caused a definite rise of pressure.

These experiments definitely show that, under the conditions

mentioned, absorption from the lungs occurs with doses of adrenalin which are ineffective when injected intramuscularly.

There was no definite relationship to be observed between the amounts of adrenalin injected into the lungs and the resultant blood pressure rise. The same dose per kilo often produced widely different rises in different animals, and smaller doses often caused greater elevations of pressure than larger ones. Table IV illustrates this and demonstrates in addition that the amount of adrenalin injected played no part.

TABLE IV.

		gm.			mm.
No. 5	Vagi intact.	1,650	0.3 cc. per kilo (0.5 cc.)	4 sec. latent period.	33 rise (107–140)
" 7	" "	1,610	0.12 cc. per kilo (0.2 cc.)	2 " " "	50 rise (100–150)
" 8	" cut.	1,980	0.25 cc. per kilo (0.5 cc.)	18 " " "	30 rise (114–144)
" 13	" "	1,790	0.25 cc. per kilo (0.4 cc.)	18 " " "	82 rise (116–198)

Blood Pressure.—The duration of the blood pressure elevation was usually less than 10 minutes; if only the effects of the first tracheal injections are considered the average is 6 minutes. Subsequent injections, however, often showed a definitely longer duration of the elevation. An illustration of this effect will be found in Experiment 7.

The character of the rise varied somewhat; it was usually more or less abrupt, the maximum being reached within 30 seconds. In other instances the maximum elevation was reached in about 1 minute. The abruptness of the rise seemed to bear some relation to the number of preceding injections, the slope becoming less steep with succeeding doses. This, however, was by no means true of all experiments, for in some all intratracheal injections gave sharp rises of pressure. Vagus pulses were often observed, but their occurrence was not as frequent as when the adrenalin is administered intravenously or intramuscularly.

In a number of experiments abrupt and profound drops of blood pressure were noted. These occurred without any warning during the maximum pressure elevation, the pressure falling within a few

seconds to 20 mm. and even less. These drops lasted from 30 to 160 seconds, and were not necessarily fatal. In some instances a number of these profound drops occurred, recovery of the blood pressure taking place spontaneously after a series of convulsions. When these drops occurred, they were always associated with more or less pronounced signs of pulmonary edema. The significance of this phenomenon will be discussed in a later paper.

Site of Absorption.—In order to obtain some information regarding the site of absorption of the injected drug, a few experiments were made with intratracheal injections of India ink or suspensions of lampblack in an extremely dilute gum arabic solution. Doses of 0.3 cc. per kilo of body weight were injected slowly by syringe into the tracheal cannula. 10 seconds after the injection the medulla was destroyed by puncture, and the lungs and trachea were immediately excised and examined. The three experiments carried out gave concordant evidence: the posterior and diaphragmatic surfaces of the lower left lobe always showed a large number of discrete and confluent, irregular black spots varying from about 2 to 5 mm. in diameter. The surfaces of the other lobes showed only a few or no spots, and these were confined largely to the posterior surfaces near the hilus of the right lower and right middle lobes.

On sectioning the lungs through the trachea and bronchi, the larger part of the left lower lobe was found to be a black mass containing foam and some fluid. The right lower and right middle lobes near the hilus also contained an amount of pigment which was greater than would be expected from the surface indications. The upper lobes and the median lappet showed a few spots in the body of these divisions.

The sections of the lung containing the pigment were larger and fuller than those free from it; moreover, they contained more fluid. The distension was greater than the amount of fluid present explained, and apparently was at least partly caused by a mechanical plugging of the bronchioles and infundibular ducts by the pigment. The amount of fluid present in the tissues seemed greater than the amount injected (0.3 cc.), so that perhaps some degree of pulmonary edema also developed.

These experiments with the tracheal injection of pigment suspen-

sions thus indicate that a certain amount penetrates to the alveoli, chiefly of the left lower lobe, within less than 1 minute, and that absorption in all likelihood takes place there.

It might be thought that some absorption could take place from the mucosa of the trachea and the bronchi, and such absorption indeed does take place, at least as far as the tracheal mucosa is concerned. This absorption from the tracheal mucosa is, however, quite slow and the blood pressure rise obtained sets in very slowly. We tested the absorptive power of the tracheal mucosa in the following way. The tracheal cannula was inserted as low as possible in the neck; the upper section of the trachea was then ligated near the cannula, converting it into a sac. Injections of adrenalin were then made into this sac and the blood pressure effect was noted. In the two experiments made the first injection of adrenalin gave each time a slow and gradual rise of pressure, the latent period being respectively 150 and 52 seconds. In the first experiment the blood pressure rise equaled 59 mm. and lasted longer than 15 minutes. In the second experiment (Experiment 6) the first injection into the tracheal sac gave a rise of only 16 mm. of mercury after a latent period of 52 seconds, the rise persisting for more than 15 minutes. A subsequent repetition of the injection gave no blood pressure effect within 6 minutes. The doses of adrenalin injected were respectively 0.3 and 0.25 cc. per kilo.

These experiments show that while absorption of adrenalin does take place from the tracheal mucosa, and therefore probably also from the bronchi and bronchioles, these surfaces play only a subsidiary part as sites of absorption when adrenalin is injected into the trachea.

Section of the vagi in the neck before the intratracheal injection of adrenalin yielded interesting results. These nerves were cut in order to prevent the occurrence of those profound blood pressure drops mentioned previously, on the assumption that they were due to the well known initial effect of adrenalin upon the vagus center. However, these drops still occurred in some of the experiments after section of the vagi, and in addition, pulmonary edema and sudden death were much more frequent than in the series with vagi intact. One intratracheal injection of adrenalin often sufficed to bring on a strong pulmonary edema and even death within a few

minutes. Without entering here into a discussion of all the phenomena observed, it may be said, in general, that section of the vagi produced no noteworthy alteration in the absorptive power of the lung tissue as far as adrenalin is concerned. The variations observed in the vagotomized series fell well within the range of those seen in the normal series, though a percentage reckoning of all intratracheal injections given for the first time, shows that the vagotomized animal exhibited a shorter latent period (10 against 13 seconds), a higher blood pressure rise (56 against 46 mm.), and a longer duration of the pressure elevation (9.5 against 6 minutes). Not much stress, however, should be laid upon averages gained from only twenty-three experiments, especially in this work where pulmonary edema entered as a complicating factor in the vagotomy series.

A number of experiments were also carried out with a 0.1 per cent solution of adrenalin made from the commercial powder, a few drops of concentrated hydrochloric acid being added to the sterile saline to bring about solution. This solution, with or without the addition of chloretone, did not give as good results when injected intratracheally as the solution obtained in the open market; the absorption was slower and the resultant rise of blood pressure less marked.

A few experiments were also carried out with the sodium salt of fluorescein. Solutions of this substance, in 1 or 10 per cent strength, were injected intratracheally, the dose being 0.3 cc. per kilo of body weight. Samples of blood were then taken at regular intervals from the carotid artery, allowed to clot in small test-tubes, and the serum was examined for fluorescence. These experiments also indicated a rapid absorption though not as striking as with adrenalin; after 15 to 30 seconds the blood samples showed the first detectable green fluorescence. This fluorescence rapidly increased at first, then more slowly, reaching a maximum after a number of minutes.

DISCUSSION AND SUMMARY.

In the preceding pages we have submitted evidence which shows that a simple intratracheal injection of a solution in a normally breathing rabbit penetrates within a few seconds to the alveoli, chiefly those of the left lower lobe; that absorption is rapid and well maintained;

and that the procedure may be repeated effectively a number of times even with a substance like adrenalin which decreases absorption. It was also shown that absorption of adrenalin from the lung could be obtained at a time when double the dose given intramuscularly exerted no blood pressure effect whatever, and that absorption could still take place after the development of pulmonary edema, when there was an undoubted dilution of the injected solution with a serum-containing liquid and when a diminution of the absorptive field had occurred.

The solution injected, after reaching the alveoli, is probably largely taken up by the capillaries of the pulmonary veins. This is indicated by the great rapidity with which an intratracheal injection of adrenalin may cause a rise of blood pressure. In numerous instances, for example, the pressure began to rise less than 5 seconds after the completion of an injection, equaling and even surpassing in rapidity of effect an intramuscular injection. Absorption by the lymphatics probably plays a secondary part, an assumption rendered all themore likely if we consider that lymph nodes are interpolated in the lymphatic pulmonary path, where the bed of the lymph stream becomes greatly widened and the current slowed.

Injection into the lungs, however, offers another advantage due to the vascular arrangement of the absorbing field which could be of value therapeutically. Absorption of liquids injected into the lung probably takes place largely through the capillaries of the pulmonary veins; to a slight extent possibly through the capillaries of the bronchial veins which empty partly into the pulmonary veins, partly into the azygos veins; and probably some absorption occurs also through the lymphatics. By far the larger proportion of the absorbed material will thus be rapidly delivered to the left auricle and then to the left ventricle. At each succeeding systole, as long as absorption continues, a fraction of the drug will be driven into the coronary arteries and be able to affect the musculature of the cardiac pump. This fact ought to render the procedure of intratracheal injection a valuable method when it becomes imperative to stimulate a suddenly failing heart as promptly as possible by drugs of the digitalis group.

Intratracheal injection is perhaps better under the conditions mentioned than the intravenous route, for the surface veins cannot

always be entered with promptness and certainty even under fairly normal conditions, and in cases of cardiac weakness the difficulties will be measurably increased, while an intratracheal injection can be carried out with ease. Moreover, it is legitimate to expect that some absorption will take place from the lung alveoli as long as the heart-lung circulation persists, no matter how feebly, and that thus some of the drug will reach the heart to act on this structure itself more promptly perhaps than when the drug is administered successfully through surface veins. As far as the intramuscular route is concerned, we have shown that the intratracheal injection of adrenalin gives prompt though diminished absorption at a time when double the dose intramuscularly exerts no blood pressure effect whatever.

The technical difficulties of giving an intratracheal injection in animals are slight. Tracheotomy as practised by us in the present series of experiments is not necessary, for the injection may be given into the intact trachea without exposure of the trachea. The hypodermic needle is inserted through the skin about 1 cm. below the larynx in a slanting caudad direction; the entrance of the needle into the trachea is readily felt. The injection should not be so rapid that the injected solution fills the entire tracheal lumen, but it should flow down the sides of the trachea. If the lumen is entirely filled, an expiration may drive some of the injected liquid into the larynx causing cough. In our experiments each injection of about 0.5 cc. consumed approximately 5 seconds.

In the human subject no data are available as far as our knowledge goes, but *a priori* it would seem that an intratracheal injection is almost as simple as in the lower animals. The free hypodermic needle could be inserted into the tracheal lumen immediately below the cricoid cartilage. The needle itself should preferably be connected with the syringe by a short length of rubber tubing to minimize the danger of breaking the needle by a sudden move of the patient. The amount of the solution should not be too small, so that at least a fraction of it may reach the alveoli as promptly as possible; 3 to 5 cc. probably would suffice.

Insertion of the needle in the locality mentioned would puncture the isthmus of the thyroid, but this is of no significance, especially when the procedure is employed in cases of cardiac failure where the

gravity of the condition would warrant incurring much heavier risks than a slight bleeding from the thyroidal isthmus.

In conclusion it may be said that the incorporation of drugs by intratracheal injection, while not as generally applicable as other methods, nevertheless has advantages which warrant its use also in human therapeutics.

THE PRODUCTION OF AMYLOID DISEASE AND CHRONIC NEPHRITIS IN RABBITS BY REPEATED INTRAVENOUS INJECTIONS OF LIVING COLON BACILLI.

BY C. H. BAILEY, M.D.

From the Pathological Laboratory of the Medical School of Leland Stanford Junior University, San Francisco.)

PLATES 110 TO 115.

(Received for publication, March 14, 1916.)

The experiments here reported are a portion of a larger series in which several varieties of bacteria were used and which were undertaken in the hope of producing in rabbits by repeated intravenous injections of living bacteria, over long periods, kidney lesions which might at least approximate the chronic lesions seen in man, which are probably due to long continued septic conditions. The results in rabbits injected with *Bacillus coli communior* were so striking in the constant and pronounced production of amyloid in various organs, as well as in the resulting kidney lesions apart from the amyloid deposits, that a brief report on them and a review of previous work on the experimental production of amyloid seems appropriate.

Frisch (1) in 1877 was the first to announce the experimental production of an amyloid-like substance. He produced a keratitis in rabbits by injection into the cornea of fresh blood from a case of anthrax. He reports positive results in 4 out of 300 corneas injected. The resulting lesion gave characteristic reactions with iodine and with iodine and sulphuric acid, but the reaction with aniline dyes was lacking and the material was doubly refracting. It seems doubtful, therefore, whether the substance was amyloid.

Birch-Hirschfeld (2) observed amyloid in the spleen of a rabbit which died after 6 weeks of chronic subcutaneous suppuration produced by the injection of pus from a case of osteomyelitis of the tibia. Other similar experiments gave negative results.

Bouchard and Charrin (3) observed amyloid in two rabbits. One had been injected subcutaneously with *Bacillus pyocyaneus* and subsequently four times intravenously at intervals of several months. The animal lived about 1 year. Amyloid was found in the kidneys and also in the vessels of the heart and in the heart muscle. A second rabbit lived 34 days after injection with material from

a human tuberculous lesion and with a culture of tubercle bacilli. Amyloid was present in the kidneys; there was none in the spleen, liver, or heart.

Czerny (4) in his first paper reports the production of amyloid in the spleen and in a few vessels of the kidneys of two dogs in which he had produced subcutaneous suppuration for 10 and 13 weeks by the injection of turpentine. The material deposited gave the iodine-sulphuric and methyl violet reactions. Czerny's paper is concerned especially with the iodophilic granules of leukocytes from which he believes amyloid is formed. In a second paper (5) he reports the production of amyloid by the same method in three more dogs, in one after suppuration lasting 7½ months, and in two after 4 months.

Condorelli-Maugeri (6) in a brief communication simply states that he has produced amyloid disease in the liver and kidneys of rabbits by injection of *Bacterium termo*. As far as the writer can discover, no detailed account of his experiments has been published.

Krawkow (7, 8, 9, 10) was the first to study systematically the question in a large series of different animals. He produced chronic suppuration by the subcutaneous injection of broth cultures of *Staphylococcus aureus* into rabbits, dogs, hens, doves, and frogs. In eight of twelve rabbits he produced amyloid, mainly in the spleen, in certain instances also in the gastro-intestinal tract, liver, kidneys, and salivary glands. In dogs the results were negative after subcutaneous suppuration lasting 2 to 3 months. The results in pigeons were also negative. He found amyloid much easier of production and more extensive in hens than in other animals, three of four hens and each of four cocks giving positive results in 1½ to 2 months. In two frogs, of several dozen injected, the spleens showed traces of a substance giving a suggestive reaction, but Krawkow was not certain that it was amyloid.

Krawkow (9) also tried Czerny's method of injecting turpentine subcutaneously and also silver nitrate, both with negative results. He believes that the abscesses produced by Czerny were not aseptic. He also injected rabbits and dogs over long periods with filtrates of cultures of *Staphylococcus aureus* and also with non-filtered cultures killed at 100°C. with negative results. Amyloid was found, however, in one rabbit which in 2 months had received seventeen injections of 5 to 30 cc. each of filtrate of a broth culture of *Bacillus pyocyaneus* killed at 100°C. In this experiment amyloid was present in the spleen in the small arteries and to some extent about the capillaries. In the kidneys traces were found about the convoluted tubules, but none in the glomeruli. None was found in the liver. As pointed out by several later workers, Krawkow's results are surprising when one considers the length of time of his experiments. In two rabbits amyloid was found in the spleen 2 days after the first injection of 10 cc. of broth culture of *Staphylococcus aureus*. In a third amyloid was pronounced after 11 days, during which time two injections of 2 and 3 cc. had been given, while in other cases injected repeatedly over a period of months, it was entirely lacking.

Davidsohn (11) confirmed the observation of Krawkow of the possibility of producing amyloid by subcutaneous suppuration resulting from repeated in-

jections of *Staphylococcus aureus*. He used rabbits, mice, hens, guinea pigs, and cats. In guinea pigs and cats the results were negative; the best results were obtained with mice and rabbits. There is the same inconsistency in time and the amount of culture injected as in Krawkow's experiments. Amyloid was found in the spleen, stomach, intestines, kidneys, and rarely in the salivary glands, pancreas, and lymph nodes. In only one instance doubtful amyloid was found in the bone marrow. In the kidneys amyloid was present in patches in small arteries of the medulla and along the collecting tubules; there was no change in the glomeruli. He obtained negative results with streptococcus, *Bacillus coli*, and putrefactive bacteria. Davidsohn believes that Czerny's positive results with turpentine may have been due to bacterial contamination of the lesions.

Lubarsch (12) was at first unable to produce amyloid by Czerny's method. Later (13) he produced suppuration for 9 to 21 weeks in eight dogs by the injection of turpentine. In one case of 16 weeks' duration, he found amyloid in the spleen only. A second was doubtful, the others negative. Seven rabbits were injected repeatedly subcutaneously with living broth cultures of *Staphylococcus aureus*, the longest period being for 9½ weeks. Positive results were obtained in two cases, of 5½ and 7 weeks. Amyloid was found in the spleen only. Guinea pigs were injected with living *Staphylococcus aureus* and dead broth cultures of *Bacillus pyocyaneus*, pneumococcus, streptococcus, and Friedländer's bacillus with negative results. Lubarsch calls attention to the irregularity of results, especially as to time, of his own experiments and those of others, and concludes that no certain method of producing amyloid has been found.

In later papers Davidsohn (14, 15, 16) reviews his own work and that of others and reports the subcutaneous injection of mice with gonococcus with positive results in two cases. His experiments with bacterial toxins gave negative results. With dead bacteria, however, he obtained positive results in one mouse in which amyloid was found in the spleen, liver, kidneys, and intestines.

Nowak (17) attempted to prove and extend the work of Krawkow, and his results seem worthy of reviewing in some detail. He experimented on rabbits and hens and found it much easier to produce amyloid in the latter. He used staphylococcus, streptococcus, *Bacillus pyocyaneus*, *Bacillus coli communis*, and their sterile filtrates; bouillon inoculated with human stool and a filtrate of this; also tuberculin, fresh and sterile pus, croton oil, and turpentine. Of seven rabbits injected with living *Staphylococcus aureus*, two, of 10 and 102 days' duration, showed amyloid in the spleen only. All hens so injected showed amyloid. Two rabbits injected with living *Bacillus pyocyaneus* were negative; two hens were positive. Two rabbits injected with living *Bacillus coli* and also two hens showed no pus and no amyloid. Rabbits injected with putrefied bouillon gave negative results, while each of two hens were positive. Of the rabbits injected with sterile filtrates of cultures of various organisms, one rabbit only, which had been injected with a filtrate of a culture of *Bacillus pyocyaneus* for 60 days, showed a few nodular deposits in the spleen which gave the amyloid reaction with aniline colors. Of nine hens injected with sterile filtrates, three were positive; two of these had

been treated with filtrates of *Staphylococcus aureus* cultures, and one with a filtrate of cultures of *Bacillus coli.* Of two rabbits and two hens injected for long periods with large doses of tuberculin, one of each showed nodules in the spleen which gave a doubtful reaction. Nowak considers them negative. Of three rabbits injected with fresh pus, one, in 8 days, showed amyloid in the spleen and liver; of two hens similarly injected, one showed amyloid in the spleen and liver. Rabbits injected with sterile pus were negative; two hens were both positive. Croton oil gave negative results. Of two rabbits injected with turpentine, one showed amyloid in the spleen and liver in 201 days; each of five hens so injected showed amyloid in the spleen and liver, and two of these in the kidneys also.

Maximow (18) also obtained positive results in hens and rabbits by repeated subcutaneous injections of living *Staphylococcus aureus.* His earliest positive result was in a rabbit after 21 days.

Schepilewsky (19) produced extensive subcutaneous necrosis and suppuration by the injection of various ferments. Out of a considerable number of experiments he reports positive results in two rabbits injected with lab-ferment, in one injected with pancreatin, and in one with papayotin. The author makes the criticism of the positive results of Lubarsch and Nowak with turpentine, and of those of Krawkow, who obtained a positive result in one rabbit with *Bacillus pyocyaneus* toxin, and of Davidsohn, who obtained a positive result in one mouse injected with dead bacteria, that bacterial contamination of the suppurating lesions produced was not excluded. Davidsohn (14, 16) on the other hand, believing that amyloid cannot be produced without the action of living bacteria or their products, makes a similar criticism of Schepilewsky's results with ferments, and apparently with equal justice. The latter admits that the abscesses in one of the four positive cases were not sterile. The other three he believes were sterile, but each of these animals died of an epidemic disease of a nature not stated, and one of the three had a purulent bronchitis.

Dantschakow (20) studied the salivary glands of rabbits injected subcutaneously with living *Staphylococcus aureus.* She believes that amyloid first appears here. She found it in some cases in the 2nd week, in others not until the 6th or 7th.

Tarchetti (21) reports entirely negative results. He injected a dog subcutaneously with *Staphylococcus aureus.* When the animal had become immune and the injections no longer produced abscesses, he used turpentine. The duration of the experiment was 4 months. Three rabbits injected subcutaneously with turpentine and eight with living *Staphylococcus aureus* were negative. He concludes that repeated injections of turpentine or broth cultures of *Staphylococcus aureus* produced no amyloid in the spleen of dogs, rabbits, or guinea pigs.

Ravenna (22) concludes from many experiments that the question of experimental production of amyloidosis still cannot be considered as solved, as a means is lacking which will produce it with certainty.

Of special interest in connection with the experiments here reported are the

observations of amyloid in the liver of horses repeatedly injected with bacteria or their toxins for the production of immune serum.

Zenoni (23) first reported the presence of amyloid degeneration in diphtheria antitoxin horses, especially in the liver. The lesion produced a very friable organ and death was apt to occur from spontaneous rupture and hemorrhage.

Pease and Pearce (24) report liver necrosis and venous thrombosis in twelve horses which had been repeatedly injected subcutaneously, seven with diphtheria toxin, two with tetanus toxin, one with dysentery bacilli, and two with streptococci. A horse is also added to the series which had been injected with diphtheria toxin and killed on account of its crippled condition. Its organs were normal. Two of these horses, both injected with diphtheria toxin, showed amyloid in the liver and spleen, especially in the latter. It was not present in the kidneys, heart, or lymph nodes. They note that these two animals had developed an unusual number of abscesses at the sites of injection, apparently attributing the amyloid formation to this fact.

Lewis (25) reports that amyloid degeneration of the liver and less frequently of the spleen is produced in a majority of horses by the routine injection of diphtheria toxin and repeated bleeding, extending over a period of 3 years. The horses are apt to die from rupture of the liver and intraperitoneal hemorrhage. He rules out abscesses as an essential condition and also the repeated bleeding, and concludes that the crude toxin is the factor of prime importance in the production of amyloid.

Schoukewitch (26) reports that repeated injections of large doses of *Bacillus pestis* produce amyloid degeneration of different organs in horses and that pus formation is not essential.

Markus (27) observed amyloid in the liver of horses injected with dysentery bacilli.

EXPERIMENTAL PART.

In the following experiments rabbits were injected in the ear vein with beef extract broth cultures of colon bacillus, transferred from stock cultures on beef extract agar. The rabbits were injected, generally every 2 to 4 days, with 1 cc. of a 24 to 48 hour culture, though older cultures were occasionally used. Colon S 232 and Colon S 244 were strains of *Bacillus coli communior* obtained in pure culture from surgically removed pyonephrotic kidneys. Colon B was also *Bacillus coli communior* isolated from a normal stool.

Frozen sections of tissues fixed on Orth's fluid were stained with hematoxylin and Sudan III and also by Van Gieson's method. Sections of paraffin-imbedded tissues were also stained by Van Gieson's method. Tissues were stained for amyloid with iodine, iodine and sulphuric acid, methyl violet, and gentian violet. As has

been previously mentioned, the finding of amyloid in these rabbits was unexpected. The hyaline bodies in the kidney glomeruli and other glomerular and parenchymatous changes had been noted, but that the bodies in the glomeruli were of amyloid nature was not recognized until a majority of the rabbits had come to autopsy and pieces of their organs had been fixed in Orth's fluid only. Consequently it was generally possible to try the various reactions only under the relatively unfavorable conditions of previous fixation in Orth's fluid. In one rabbit, No. 15, the reactions were tried on fresh and alcohol-fixed tissues; in Rabbits 1, 3, and 7, on alcohol-fixed tissues; in all others, on tissues fixed in Orth's fluid only. The reactions were tried under these conditions on the spleen, liver, and kidneys of all rabbits. Pieces of other organs, however, which are of interest in connection with amyloidosis had not been preserved in most cases, and it is therefore impossible to report the presence or absence of amyloid degeneration in other situations except in a few instances. Table I shows the strain of bacteria used, the duration of the experiment in days, the number of injections, and the presence or absence of amyloid in the spleen, liver, and kidneys. Positive findings in other organs will be mentioned later.

TABLE I.

Rabbit No.	Bacteria.	Duration of experiment.	No. of injections.	Amyloid.
		days		
1	Colon S 232	21	7	None.
2	" S 232	26	9	"
3	" S 232	30	11	"
4	" S 232	30	10	"
5	" S 244	59	8	"
6	" S 232	61	15	"
7	" S 232	63	19	"
8	" S 232	88	23	Spleen, liver, kidney.
9	" S 232	98	25	"
10	" S 232	102	25	"
11	" S 232	113	29	" liver, kidney.
12	" B	115	27	" kidney.
13	" S 232	116	36	" "
14	" S 232	142	30	" "
15	" S 232	145	47	" liver, kidney.

Complete autopsies were performed on all the animals, which, however, will not be reported in detail, as the gross pathological findings were few. Suppurative lesions were lacking with the following exceptions: Rabbit 3 had an abscess about 1.5 cm. in diameter in the upper anterior mediastinum, and Rabbit 14 had a few small nodular areas of consolidation in the lungs, each about 3 mm. in diameter with central softening. All the rabbits lost much in weight, emaciation being extreme in the older cases. The livers were of about normal size and no change in consistency was noted except in No. 15, in which the organ was definitely firmer than normal but more friable. All the spleens were somewhat enlarged; in No. 14 the organ was about four times normal size, in No. 9 about three times, in Nos. 1 and 11 about twice; in the others the enlargement was slight. Beyond the enlargement there was no marked change in appearance or consistency. The gross appearance of the kidneys was not striking. In all the animals they were definitely opaque, in many very pale, and in some apparently swollen. Small, slightly depressed, purple or gray surface scars were present in many instances, in some being moderate in number, in most very few. None were present in Nos. 1, 2, 3, 4, 5, and 7. They had the same appearance as the spontaneous scars in rabbits, and it is difficult to decide whether they are to be ascribed to the treatment or to a preexisting spontaneous nephritis. The fact that they were absent in the experiments of shorter duration would favor the former view. The number and severity of the scars when present, however, definitely bore no relationship to the length of time the rabbit had been injected, the scars in Nos. 6, 9, and 10 being more numerous than in the older rabbits, in which they were very few and slight.

Microscopical examination of the spleens of the earlier rabbits, Nos. 1 to 7 inclusive, showed a large amount of hematogenous pigment collected in large phagocytic cells in the sinuses of the pulp. In some there was a slight diffuse fibrosis. Otherwise the spleens were normal. In Rabbits 8 to 15 the hematogenous pigment was also present and in all there was a fibrosis. All showed about the Malpighian bodies a homogeneous hyaline substance which in the more marked cases, Nos. 8, 11, 12, and 13, encroached on these structures (Fig. 4) and almost entirely replaced them, only a small

collection of lymphoid cells remaining grouped about the central artery. A moderate amount of nuclear detritus was present in this material, and in No. 8 numerous fairly large hemorrhages were present in the midst of the hyaline material, which had largely replaced the Malpighian bodies. Similar hyaline material was also seen in some animals in the reticulum of the pulp. In Rabbits 14 and 15 the fibrosis and hyaline formation, though more marked about the Malpighian bodies, was also present through the reticulum of the pulp (Fig. 5). Stained with methyl violet or gentian violet, this hyaline material, both about the Malpighian bodies and in the pulp, gave a typical red amyloid reaction in all cases (Fig. 1). It appeared in the form of small irregular masses which usually were quite homogeneous, but sometimes suggested a finely granular structure. When present in the reticulum of the pulp it had a definite fibrillar appearance. With iodine no reaction was obtained even in the fresh and alcohol-fixed tissues of No. 15. With iodine followed by sulphuric acid the larger collections about the Malpighian bodies gave a pale but definite greenish color. These reactions will be considered later. Large phagocytic cells were quite numerous in the spleen of these rabbits, and in No. 15 fairly numerous giant cells were present, having many nuclei and often including masses of the amyloid (Fig. 1). These cells have been previously noted by Krawkow (7).

All the livers showed a definite but usually moderate increase of periportal connective tissue and also an infiltration with round cells in these areas. It is difficult to decide whether this is a spontaneous cirrhosis or is due to the treatment. There were also present in all the animals a varying number of small focal necroses situated indiscriminately in the lobules. In the earlier ones these were infiltrated with round cells; in the later ones they were often replaced by connective tissue. There was considerable granular degeneration of the liver cells in these rabbits and also pyknosis of nuclei. In No. 13 numerous large central necroses were present with much fibrin and some hyaline degeneration but little cellular infiltration. The livers of Rabbits 8, 11, and 15 showed between the walls of the capillaries and the trabeculæ of liver cells a layer of hyaline material with atrophy and granular degeneration of the intervening liver cells. This was present in al lobules, but in No. 8 was seen only in the peripheral and middle

zones of the lobules, while in the other two it was fairly uniform throughout. With iodine and iodine followed by sulphuric acid, no reaction was obtained. With methyl violet and gentian violet this material gave a typical amyloid reaction. With these stains the substance appeared usually in a definite granular form (Fig. 2), but sometimes a fine fibrillar structure was apparent. The necroses with hyaline degeneration described in No. 13 gave no reaction. No amyloid was found in the walls of the larger vessels.

The kidneys of these rabbits deserve special attention, since they showed not only amyloid deposits but also other lesions definitely due to the bacterial injections. Degenerative changes were present in the tubular epithelium in all cases. Frozen sections stained with Sudan III showed a moderate fatty degeneration affecting principally the convoluted tubules. The fatty degeneration was, however, more marked in the earlier than the later cases. The was a definite granular degeneration, also most marked in the convoluted tubules, and some necrosis of the epithelium with pyknosis of nuclei, lack of nuclear staining, and desquamation. The large granules seen in the epithelium of the proximal convoluted tubules in chronic parenchymatous nephritis in man were not observed. Casts were present in all kidneys except those of Nos. 1 and 2, and in Nos. 13, 14, and 15 they were numerous. No glomerular changes, other than hyperemia, were noted in Rabbits 1 to 7, while all the others showed evidences of a subacute and chronic glomerulitis. The lesions consisted of fibrous and hyaline thickening of the vascular loops and occasionally of the glomerular capsules, protoplasmic, cellular, and fibrous adhesions between the tufts and capsules, areas of necrosis in the tufts, and hyaline bodies containing a few pyknotic nuclei and some nuclear débris. These lesions varied in number and degree in the different rabbits. Adhesions were present in Nos. 8 to 15 inclusive, being, however, few in Nos. 9 and 12 but numerous in the others, especially Nos. 13 and 14 (Fig. 6). Almost every glomerulus in No. 13 showed adhesions. Some of these adhesions were fine protoplasmic unions between tuft and capsule (Fig. 7), others large and cellular or fibrous (Figs. 7 and 8). Fibrous and hyaline thickening of the vascular loops was present in Nos. 8, 11, 12, 13, 14, and 15, with necroses and large amyloid bodies in some instances (Figs. 7, 8, and 9). The fre-

quent occurrence of localized interstitial scars in the kidneys of rabbits unfortunately renders the interpretation of interstitial lesions in these animals difficult. As has been already mentioned, most of the rabbits which withstood the injections longest showed a few small scars in the gross. There were usually only two or three such scars on a kidney. These scars have been disregarded as perhaps due to a preexisting spontaneous nephritis. In most of the kidneys, however, there were present small collections of round cells, and in Nos. 13, 14, and 15 there was a slight, rather diffuse, cellular thickening of the interstitial tissue. It is believed that these lesions were produced experimentally.

Six of the kidneys, as shown in the table, gave a typical amyloid reaction with methyl violet. The amyloid was present in the glomeruli as small and large nodular collections (Fig. 3) and also in the medulla where in some instances it was situated only about the small capillaries in small localized areas; in others it involved the entire interstitial tissue, in similar small nodular areas, here surrounding both vessels and tubules. No amyloid was noted in the larger vessels. Here as in other organs it was difficult to convince one's self definitely of the morphology of the amyloid. In certain instances the large deposits in the glomeruli seemed to have a definite fibrillar structure with a frayed out appearance at the edges, especially noticeable in the alcohol-fixed tissue of No. 15. In some of the other cases these bodies appeared quite homogeneous (Fig. 3). With iodine no reaction was obtained even with frozen sections of the fresh tissue of No. 15. With iodine and sulphuric acid the larger bodies in the glomeruli, like the deposits in the spleen, gave a pale but definite greenish reaction.

By reference to the table one notes that amyloid was present in the spleen of all rabbits, eight in number, which were injected for a period of 88 days or longer. These eight rabbits showed glomerular and other lesions in the kidneys, and in six amyloid was present. The livers of three contained amyloid. Organs other than the spleen, liver, and kidneys were examined for amyloid in only a few instances. The stomach, small intestine, large intestine, heart, adrenal, and submaxillary gland in No. 3 gave no reaction. The small intestine, large intestine, heart, and submaxillary gland in No. 7 gave no

reaction. The stomach and small intestine of No. 15 showed typical deposits in small scattered areas in the mucosa only; small irregular homogeneous bodies in the bone marrow gave a doubtful reaction with methyl violet; the heart and adrenals showed none.

DISCUSSION.

It seems justifiable to regard the deposits described in these animals as amyloid in spite of the absence of the iodine reaction. It was possible to try the reaction on fresh or alcohol-fixed tissues in only one, No. 15, of the eight rabbits which showed deposits. There is a possibility that the reaction might have been obtained in some of the other animals under these conditions. It is the consensus of opinion, however, of those who have studied the question carefully, that the reaction with aniline colors is the more typical and that the iodine and iodine-sulphuric reactions are sometimes lacking. Davidsohn (11) and others believe that these reactions represent different stages in the development of amyloid, the reaction with aniline colors appearing earliest and being present throughout, the iodine reaction appearing next, and that with iodine and sulphuric acid representing the latest stage. Krawkow (10) and Maximow (18) report results similar to the above with the amyloid experimentally produced by them in animals. Krawkow (10) found the reaction with methyl violet constant, but obtained the iodine reaction in only a few instances, and then only on fresh or alcohol-fixed tissues, and in the latter it disappeared after 24 hours' fixation. Similar absence of the iodine reaction in amyloid deposits in man has been reported by Hansemann (28), Krawkow (10), and others. Krawkow (10) believes that these reactions do not represent a difference in chemical composition of the substance, but are due to a difference in physical conformation of the older deposits. The presence of the iodine-sulphuric reaction in the absence of the simple iodine reaction appears from the record of previous experiments to be exceptional. The possibility suggested itself that the green color obtained in these cases might be due to the presence of cholesterol in these degenerated areas. The large deposits in the kidneys and spleen, however, give no suggestion of a fat reaction with Sudan III, as one would expect were

the cholesterol present in any considerable amount in lipoid form; and the fact that the reaction was also obtained on specimens imbedded in paraffin would seem to rule out cholesterol as the responsible factor.

Scrapings of the fresh liver, spleen, and kidneys of Rabbits 3, 6, 8, 11, 13, and 14 were examined with the polarizing microscope for anisotropic fat. This examination is of interest on account of the reports of the presence of doubly refracting lipoids in human nephritis, particularly in the chronic parenchymatous type which these kidneys so closely resemble. The results were negative except in the liver of No. 6 and the kidneys of No. 8, in both of which a moderate number of small anisotropic droplets were present.

One notes that in the spleen there is an association of amyloid formation with evidences of blood destruction, cell necrosis, and fibrosis. Also in the glomeruli of the kidneys one sees necrosis and fibrosis associated with the deposition of amyloid. In the liver this association is not so evident. It is not justifiable to draw definite conclusions as to a possible relationship between amyloid formation and any one of these other phenomena. While the cell destruction may in part be the result of the deposition of amyloid, or the two may be mutually independent results of the toxic action, we believe that the possibility of a genetic relationship between cell necrosis and the formation of amyloid should not be too readily dismissed. Mallory's views are worthy of attention in this regard. He states (29) that amyloid in man when first deposited is finely fibrillar but soon becomes homogeneous and that it is not a product of degeneration of cell or fibril, nor is it something filtered out of the blood-like serum, but it is an abnormal product of the fibroblast. His argument that it is more reasonable to regard amyloid as the product of the fibroblast, which normally produces fibrils, than of the endothelial cell which does not, is forcible. He also calls attention to the fact that chondroitin sulphuric acid is a constituent of some of the normal products of these cells; namely, elastic fibers and chondromucin. As between its formation by endothelial cells and fibroblasts, these facts are certainly in favor of the latter. As previously noted, its fibrillar appearance in the spleen and kidneys of these rabbits is sometimes quite evident. This fibrillar appearance, however, does not seem to the writer good evidence that it is produced

as such by either fibroblasts or endothelial cells. The variation in the morphological appearance of the amyloid in different situations in these rabbits has already been noted. One is also impressed in reviewing the literature with the variations in appearance as described by different authors, some believing it to be fibrillar or homogeneous, others granular, and others crystalline. Of the various theories advanced as to the nature of this substance, the idea that it is a homogeneous, semisolid, infiltrating substance would seem to explain these variations in morphological appearance better than any other, variations being produced, as they are particularly apt to be in colloidal materials, by postmortem changes or the processes of fixation, and being less easily produced in older deposits, perhaps because of greater inspissation. If such a substance were produced or deposited in narrow tissue spaces, it might well appear in fixed specimens as thin sheets or fibrils. With atrophy of intervening collagen fibrils, which Mallory describes as occurring following the appearance of the amyloid fibrils, the infiltrating material might well fuse to form the homogeneous masses of the later stages.

Living bacteria only were tried in the experiments here reported. That the injection of dead colon bacilli alone might result in amyloid deposits is suggested by the results of Davidsohn (16) on a mouse injected with dead bacteria, and that toxins or decomposition products of the culture media might produce it is suggested by the results of Krawkow (7) and Nowak (17), each on a rabbit injected with filtrate of a culture of *Bacillus pyocyaneus* and by those on horses immunized with diphtheria toxin. These are questions not answered by these experiments. That it could be due to the normal constituents of beef extract broth would seem improbable. That this is not the case is evidenced by Rabbit 6 which had received twenty-five intravenous injections of sterile broth in a period of 101 days before the bacterial injections were begun. The period of injection of sterile broth was thus not as long as that of some of the rabbits which showed amyloid, but longer than in two of the rabbits, and about the same as that of a third which showed definite amyloidosis.

The question as to whether the ability to produce amyloidosis is a common property of all pathogenic bacteria or is confined to certain species or classes, is of considerable practical interest and also

one which has not been answered. From a study of previous work it seems evident, in spite of conflicting reports, that subcutaneous suppuration produced by living *Staphylococcus aureus* in hens and rabbits may, especially in the former, ultimately result in the formation of amyloid deposits. The positive results reported with other organisms are so few and the variety of animals on which these results were obtained is so great, that it is difficult to draw conclusions in this regard. The criticism made of the experiments in which subcutaneous suppuration has been produced by turpentine, ferments, and bacterial toxins,—that bacterial contamination was not excluded,—seems justified. The difficulty of keeping such subcutaneous lesions uncontaminated, especially with staphylococcus which we know will produce amyloid, over periods of weeks or months is obvious. Attention has already been called, in reviewing the literature, to the contradictory reports as regards the possibility of producing amyloidosis by subcutaneous suppuration and also to the inconsistency, as far as time is concerned, in the experiments of the various investigators who have reported positive results. In contrast with previous work the constant results here reported suggest the importance of bacteriemia in the production of amyloid and afford a possible explanation of the varying results in previous experiments, that in some cases bacteriemia resulted while in others the infection remained localized. Certain results reported, particularly those on horses, indicate that a toxemia is sufficient, without actual bacteriemia.

The author has previously stated that the experiments here reported with colon bacilli are a part of a larger series in which various bacteria were used. The only rabbit injected with other varieties of bacteria which lived 88 days or longer (that is, longer than the shortest period in which amyloid was produced with colon bacilli) was one injected with *Bacillus typhosus*. This animal received forty injections of 1 cc. each of broth culture in a period of 125 days. Another rabbit received twenty-three injections of 1 to 2 cc. each in a period of 84 days. Neither of these rabbits showed amyloid deposits. One would not be justified from these two instances in drawing definite conclusions, yet the results would seem to indicate that at least there is a marked difference in the facility with which amyloid is produced by different organisms. The principal other bacteria used were

various strains of streptococci. Amyloid was not produced, but inasmuch as the duration of the longest experiments was 87 days (ten injections) and 78 days (twenty-two injections) comparative conclusions in these instances also are not warranted. If experimental investigation should prove that amyloid is produced more easily by some varieties of organisms or their products than by others, a careful investigation of the numbers and varieties of bacteria present in pulmonary cavities in chronic tuberculosis would also be of interest as possibly affording an explanation of the recognized fact that amyloidosis is extreme in some cases of chronic tuberculosis while entirely lacking in others of equal duration and extent of lesions. The explanation which has been offered of individual and racial susceptibility seems inadequate to explain the marked differences seen in this regard. At least the explanation is one to which we should not resort until the above possibility has been excluded.

SUMMARY.

The repeated intravenous injection of rabbits with living *Bacillus coli communior* over long periods has resulted in the formation of amyloid deposits in the spleen, liver, and kidneys. Suppurative lesions were not present in most cases and therefore not a factor in its production. The results have been constant in that amyloid was found in all rabbits, eight in number, which were injected over a period of 88 days or more. Eight rabbits showed amyloid in the spleen, six of these in the kidneys also, and three in the liver.

The kidneys of these eight rabbits also showed as a result of the injections a subacute and chronic glomerulitis, parenchymatous degeneration, some interstitial infiltration with round cells, and a slight cellular proliferation of connective tissue, thus resembling the chronic parenchymatous nephritis of man which is so commonly associated with amyloid disease.

In conclusion I wish to express my appreciation of the advice and assistance of Dr. Ophüls throughout this work.

BIBLIOGRAPHY.

1. Frisch, A., Ueber eigenthümliche Produkte mykotischer Keratitis mit der Reaction des Amyloids, *Sitzungsb. d. k. Akad. d. Wissensch. Math. naturw. Cl., 3te Abt.*, 1877, lxxvi, 109.
2. Birch-Hirschfeld, F. V., Lehrbuch der pathologischen Anatomie, Leipzig, 1882, i, 45.
3. Bouchard and Charrin, Dégénérescence amyloïde expérimentale, *Compt. rend. Soc. de biol.*, 1888, v, 688.
4. Czerny, A., Zur Kenntniss der glycogenen und amyloïden Entartung, *Arch. f. exper. Path. u. Pharm.*, 1893, xxxi, 190.
5. Czerny, A., Ueber die an Thieren experimentell hervorgerufene Amyloidentartung, *Centralbl. f. allg. Path. u. path. Anat.*, 1896, vii, 282.
6. Condorelli-Maugeri, A., Ueber die Aetiogenese der Amyloiddegeneration, *Centralbl. f. allg. Path. u. path. Anat.*, 1894, v, 417.
7. Krawkow, N.-P., Ueber bei Thieren experimentell hervorgerufenes amyloid, *Centralbl. f. allg. Path. u. path. Anat.*, 1895, vi, 337.
8. Krawkow, N.-P., De la dégénérescence amyloïde et des altérations cirrhotiques provoqués expérimentalement chez les animaux, *Arch. de méd. expér.*, 1896, viii, 106.
9. Krawkow, N.-P., De la dégénérescence amyloïde et des altérations cirrhotiques provoqués expérimentalement chez les animaux, *Arch. de méd. expér.*, 1896, viii, 244.
10. Krawkow, N.-P., Beiträge zur Chemie der Amyloidentartung, *Arch. f. exper. Path. u. Pharm.*, 1898, xl, 195.
11. Davidsohn, C., Ueber experimentelle Erzeugung von Amyloid, *Virchows Arch. f. path. Anat.*, 1897, cl, 16.
12. Lubarsch, O., Beiträge zur Histologie der von Nebennierenkeimen ausgehenden Nierengeschwülste, *Virchows Arch. f. path. Anat.*, 1894, cxxxv, 149.
13. Lubarsch, O., Zur Frage der experimentellen Erzeugung von Amyloid, *Virchows Arch. f. path. Anat.*, 1897, cl, 471.
14. Davidsohn, C., Die Rolle der Milz bei der Amyloiderkrankung, *Verhandl. d. deutsch. path. Gesellsch.*, 1904, vii, 39.
15. Davidsohn, C., Allgemeine pathologische Morphologie und Physiologie. I. Arbeiten über Amyloid und Hyalin, 1899–1907, *Ergebn. d. allg. Path. u. path. Anat.*, 1908, xii, 424.
16. Davidsohn, C., Untersuchungen über die Aetiologie des Amyloids, *Virchows Arch. f. path. Anat.*, 1908, cxcii, 226.
17. Nowak, J., Experimentelle Untersuchungen über die Aetiologie der Amyloidosis, *Virchows Arch. f. path. Anat.*, 1898, clii, 162.
18. Maximow, A., Ueber die experimentell hervorgerufene Amyloid-Entartung der Leber, *Virchows Arch. f. path. Anat.*, 1898, cliii, 353.
19. Schepilewsky, E., Experimentelle Beiträge zur Frage der amyloiden Degeneration, *Centralbl. f. Bakteriol., 1te Abt.*, 1899, xxv, 849.

20. Dantschakow, W., Über die Entwicklung und Resorption experimentell erzeugter Amyloidsubstanz in den Speicheldrüsen von Kaninchen, *Virchows Arch. f. path. Anat.*, 1906, clxxxvii, 1.
21. Tarchetti, C., Untersuchungen über experimentelle amyloide Entartung,
22. Ravenna, E., Ueber die experimentell erzeugte Amyloiddegeneration, *Centrabl. f. allg. Path. u. path. Anat.*, 1903, xiv, 677.
23. Zenoni, C., Toxische Amyloidosis bei der antidiphtherischen Immunisation des Pferdes, *Centralbl. f. allg. Path. u. path. Anat.*, 1902, xiii, 334.
24. Pease, H. D., and Pearce, R. M., Liver Necrosis and Venous Thrombosis in Horses Actively Immunized with Diphtheria and Tetanus Toxins and with Streptococci and Their Products, *Jour. Infect. Dis.*, 1906, iii, 619.
25. Lewis, P. A., Hemorrhagic Hepatitis in Antitoxin Horses, *Jour. Med. Research*, 1906, xv, 449.
26. Schoukewitch, J., De la dégénérescence amyloïde chez les chevaux soumis aux injections répétées de bacilles pesteux, *Arch. d. Sc. biol.*, 1907, xii. 190.
27. Markus, H., Over amyloide Degeneratie van de Lever bij het Paard, *Tijdschr. v. veeartsenijk. Maanbl.*, 1904–05, xxxii, 99.
28. Hansemann, Amyloider Degeneration nach Syphilis, *Deutsch. med. Wchnschr.*, 1893, xix, 582.
29. Mallory, F. B., The Principles of Pathologic Histology, Philadelphia, 1914, 102–107.

EXPLANATION OF PLATES.

Plate 110.

Fig. 1. Rabbit 15. Spleen. A portion of a Malpighian body is shown which was surrounded and partially replaced by amyloid. A giant cell is seen, at the upper end of the picture, with amyloid inclusions. Camera lucida water color drawing by Dr. F. E. Blaisdell. Zeiss obj. E., oc. 1. Stained with methyl violet.

Fig. 2. Rabbit 15. Liver. The picture shows a considerable deposit of amyloid between the capillary walls and liver cells with granular degeneration and atrophy of the latter. The amyloid has a finely granular appearance. Camera lucida water color drawing by Dr. F. E. Blaisdell. Zeiss obj. E., oc. 1. Stained with gentian violet.

Plate 111.

Fig. 3. Rabbit 14. Kidney. The upper glomerulus was of homogeneous appearance with loss of normal structural detail and contained many pyknotic nuclei and a large homogeneous appearing amyloid body. The lower glomerulus showed a general thickening of the vascular loops with two small amyloid nodules. There were adhesions in both cases between tuft and capsule. Camera lucida water color drawing by Dr. F. E. Blaisdell. Zeiss obj. E., oc. 1. Stained with methyl violet.

PLATE 112.

FIG. 4. Rabbit 12. Spleen. A Malpighian body is shown surrounded and encroached upon by amyloid formation; also thickening of and deposition of amyloid in the reticulum of the pulp. Microphotograph. Bausch and Lomb obj. $\frac{2}{3}$, oc. 1.

FIG. 5. Rabbit 14. Spleen. The photograph shows a fibrosis affecting the Malpighian body and also the reticulum of the pulp. There is some amyloid about the Malpighian body, but it is relatively more pronounced in this case in the reticulum of the pulp. Microphotograph. Bausch and Lomb obj. $\frac{2}{3}$, oc. 1.

PLATE 113.

FIG. 6. Rabbit 13. Kidney. The photograph shows eight glomeruli in each of which there are adhesions between tuft and capsule. Two glomeruli, the one at the top of the picture in the middle and also that at the bottom, show large amyloid bodies. Microphotograph. Bausch and Lomb obj. $\frac{2}{3}$, oc. 1.

PLATE 114.

FIG. 7. Rabbit 13. Kidney. A higher magnification of the glomerulus seen at the top of Fig. 6. Note the fine protoplasmic and large cellular adhesions, the hyaline body, and the swelling and slight proliferation of the lining cells of the capsular space. In an adjacent section of this same glomerulus the hyaline body gave a typical amyloid reaction with methyl violet. Microphotograph. Bausch and Lomb obj. $\frac{1}{6}$, oc. 1.

PLATE 115.

FIG. 8. Rabbit 13. Kidney. A glomerulus from the same rabbit but not in the field of Fig. 6. Note the extensive adhesions and the large hyaline body containing some nuclear débris. In an adjacent section the hyaline body in this glomerulus gave a typical amyloid reaction with methyl violet. Microphotograph. Bausch and Lomb obj. $\frac{1}{6}$, oc. 1.

FIG. 9. Rabbit 15. Kidney. Note the large body in the glomerulus with a few pyknotic nuclei and some nuclear débris. In an adjacent section of this same glomerulus the entire body gave a typical amyloid reaction, the material appearing in a distinctly fibrillar and finely granular form. Microphotograph. Bausch and Lomb obj. $\frac{1}{6}$, oc. 1.

BACTERIOLOGICAL AND EXPERIMENTAL STUDIES ON GASTRIC ULCER.

By HERBERT L. CELLER, M.D., and WILLIAM THALHIMER, M.D.,*

(*From the Pathological Laboratory, Mount Sinai Hospital, New York.*)

(Received for publication, March 8, 1916.)

Many investigators have attempted to produce chronic gastric ulcers experimentally. These experiments have been performed on various species of animals, and a great variety of methods has been used in causing the original injury to, or defect in the gastric mucosa. All these attempts have failed, and the continuity of the gastric mucosa was always restored by prompt healing unless the procedure caused a complete stoppage of the blood supply to a part of the stomach. This was followed in some instances by infarction and by perforation and its sequelæ.

Recently Rosenow[1] has published some interesting investigations on the production of acute and chronic gastric ulcers in rabbits and dogs. His full report has not yet appeared. He isolated anhemolytic streptococci, so called *Streptococcus viridans* or *mitis*, from 96 per cent of a series of gastric ulcers removed from human subjects at operation. Recently isolated cultures were injected intravenously into animals, and in 60 per cent gastric ulcers were found. In a few animals which were allowed to live for a considerable time after their inoculation, in some instances several months, chronic ulcers were found at autopsy. From these results Rosenow has reached certain conclusions which may be briefly summarized as follows:

1. Anhemolytic streptococci can be recovered by a special technique from practically all gastric ulcers removed at operation.
2. The streptococci from this source possess a specific affinity for the stomach which enables them to localize in this organ, when recently isolated cultures are injected intravenously into animals.
3. About 60 per cent of the animals inoculated in this manner develop gastric

---

* This work was done under the tenure of a George Blumenthal, Jr., Fellowship.

[1] Rosenow, E. C., and Sanford, A. H., *Jour. Infect. Dis.*, 1915, xvii, 219; *Jour. Ind. State Med. Assn.*, 1915, viii, 458; *Jour. Am. Med. Assn.*, 1915, lxv, 1687.

ulcers. Other lesions also occur following these inoculations, but with less frequency.

4. Streptococci can be recovered from these experimental ulcers and can be demonstrated histologically. They reach this location by the blood stream and are deposited in the capillaries of the gastric mucosa.

5. These streptococci are identical with those inoculated.

6. Anhemolytic streptococci are, therefore, the cause of gastric ulcers in man, and these organisms reach the stomach by a hematogenous route.

The following experiments were undertaken following Dr. Rosenow's presentation of his investigations in New York in April, 1915. The technique which we employed in isolating and injecting the streptococci is identical with his. We are indebted to Dr. Rosenow for having furnished us with a description of his technique before the publication of his work.

Material.

Eight chronic gastric ulcers removed at operation from human subjects were examined bacteriologically and histologically We have also studied one ulcer occurring at the ostium of a gastrojejunostomy. The results obtained from these examinations and the animal experiments performed with the organisms recovered from these ulcers form the basis of the following report. Although the number of specimens studied has not been large, the consistency of the results obtained by all three methods of investigation seems to us to justify the present communication.

The eight gastric ulcers studied were all of the chronic indurated type. All the cases from which they were removed had typical gastric symptoms which varied in duration from 2 months to 8 years. Two of the ulcers were located at the pylorus and six on the lesser curvature.

On examination of the stomach at operation the ulcer-bearing area was found thickened and indurated and either a defect in the mucosa or a crateriform ulcer was felt.

Brief descriptions of the gross appearance of the specimens examined follow.

A. Small piece of ulcer removed. Ulcer felt crateriform to operator.

C. Funnel-shaped ulcer of the greater curvature, near pylorus, 1.5 cm. in width, with firm fibrotic margins.

D. Ulcer of the lesser curvature, 1.5 cm. in width, extending through the entire gastric wall into the gastrohepatic omentum. Edges overhanging, firm, and fibrotic.

E. The same appearance as D.

G. Ramifying, irregular ulcer involving the lesser curvature, the entire pyloric antrum, and the first cm. of the duodenum. It has a punched out appearance with sharp edges, not overhanging; the base extends into the muscularis.

J. At the middle of the lesser curvature of the pyloric antrum there is an ulcer 2 cm. in width, perforating entirely through the stomach wall into the gastrohepatic omentum, with sharp, firm edges that do not overhang.

L. Round, punched out ulcer, 1 cm. in width, with firm edges which slightly overhang. The ulcer extends almost through the entire wall of the stomach.

M. The specimen consists of a segment of the middle 7 cm. of the stomach. The middle of the posterior wall is occupied by a perforated ulcer, 3 cm. in diameter, with sharp edges.

Gastrojejunal Ulcer.—This specimen was removed from a patient on whom 3 years previously a posterior gastrojejunostomy by the Murphy button method had been performed, together with a pyloric exclusion by the string method for a perforated duodenal ulcer situated just beyond the pylorus.

Operation and Specimen.—At the site of the former duodenal ulcer there is an indurated area. The string is still in place and the pylorus almost completely closed, barely admitting the tip of the little finger. The gastrojejunostomy opening is considerably stenosed and admits only the tip of the little finger. The edge of the ostium appears soft and normal over about two-thirds of its circumference. The remaining one-third feels indurated and the serous surface over this is bright red in color and shows a few very recent, fibrinous adhesions between the jejunum and the transverse mesocolon. The anastomosis between the jejunum and the stomach was separated with difficulty. Beneath the reddened portion of the ostium was found what appeared to be a definite and somewhat indurated ulcer of the stomach, 1.5 cm. in size. This portion was resected and a new gastrojejunostomy was performed at the same site, by the suture method.

Methods.

Immediately after the operation pieces of ulcer were removed for culture and the remainder of the material was fixed in 70 per cent alcohol. Some of the pieces were incubated in deep tubes of glucose-serum-bouillon for 24 and 48 hours and then fixed in alcohol. Sections were prepared by both the paraffin and celloidin methods and stained with hematoxylin and eosin, Giemsa and Unna-Pappenheim stains, and by the Gram-Weigert method.

Formalin fixation was not used as this interferes with the demonstration of bacteria. The aniline oil, gentian violet used in the Gram-

Weigert method was always filtered through Berkefeld filters to remove any bacteria present which might be deposited on the section and lead to confusion.

Microscopical Studies.

Microscopical examination reveals a great similarity in the pathological processes in the specimens. All the ulcers show certain prominent characteristics. The edge of the ulcer is sharp, as though cut off, even when it is overhanging. The mucosa at the edge shows only a superficial degeneration and a moderate grade of chronic inflammation consisting of congestion, edema, and diffuse infiltration with mononuclear leukocytes, plasma cells, and a few polymorphonuclear leukocytes. The glandular structures at the edge of the ulcer show slight degenerative changes of their cells. Some of the glandular acini or crypts also have the appearance of being cut off and do not reach the surface of the mucosa but terminate at the edge of the ulcer. Whenever found, the cells of the acini are contained within their basement membrane and show no tendency to hyperplasia or malignant transformation. The lining, *i. e.*, the surface of the ulcer, consists of a narrow layer from one-eighth to one-third the diameter of a low power field of the microscope in thickness and resembles very much the degenerating tissue which is found situated in tuberculous lesions between the areas of caseation and the surrounding tuberculous granulation tissue. The layer of degenerating tissue fuses with or is transformed into dense fibrous tissue, although the line of demarcation is clearly seen. The fibrous tissue extends for a considerable distance, in places as much as 1 cm., into the submucosa and muscularis. It has the typical characteristics of scar tissue and seems to form a wall beneath the ulcer. Some of the ulcers have completely penetrated the stomach wall, but the edges are always covered by the above structures and the bare muscularis is never exposed. The connective tissue septa which lie between the muscle bundles and are accompanied by blood vessels, are increased in size by fibrous tissue and dense infiltration with mononuclear leukocytes. Similar areas of fibrosis and infiltration are found beneath and in the serosa.

Occasionally, but only rarely, prolongations of the degenerat-

ing surface layer, several mm. in size, are found in the dense fibrous tissue layer, but these are always completely limited by the latter.

With the special stains for bacteria, especially with the Gram-Weigert stain, organisms are found only in or upon the lining of the degenerating tissue but never in the depths of this except in the prolongations described above. The organisms usually occur in small clumps or as sparsely scattered individuals, and are not found in the dense fibrous layer, the stomach wall, the connective tissue septa, or the areas of leukocytic infiltration of the serosa. The chief organisms found are minute cocci (streptococci), peculiar forms of yeast, and various types of bacilli. The same forms are found in great numbers throughout the tissue which was incubated in bouillon before it was fixed in alcohol, but are most numerous on and near the surface of the ulcer. The types of organisms observed in the preparations from the material fixed immediately and the organisms recovered in cultures are given in parallel columns in Table I.

In all, 109 tubes were inoculated either with emulsions or pieces of ulcer. Streptococci were present in 32 tubes, occurring in pure culture in 11. 13 tubes remained sterile, and the other 64 tubes contained various types of organisms.

The streptococci were anhemolytic and had the following morphological and cultural characteristics.

Morphology.—Stained preparations. Small, Gram-positive cocci, forming short and long chains. No capsule. Hanging drop of growth in deep tubes of glucose-serum-bouillon shows long convoluted chains.

Bouillon.—Slight diffuse cloudiness; many granular clumps adherent to the sides of the tube and also deposited at the bottom as sediment.

Litmus Milk.—Acidified but not coagulated.

Gelatin.—Not fluidified.

Inulin-Serum-Peptone-Water-Medium.—Not fermented.

Bile.—No solution of organisms.

2 Per Cent Glucose-Serum-Agar.—Dry, granular, fairly profuse growth. All strains cause precipitation, most of them marked precipitation.[2]

Plain Agar.—Sparse, dry growth, mostly as minute, discrete, pearly white colonies.

---

[2] Libman, E., *Jour. Med. Research*, 1901, i, 84.

TABLE I.

Ulcer.	Types of organisms found in sections of ulcers.	Organisms recovered in cultures from ulcers.
A	Few groups of round bodies, suggestive of cocci, upon surface of ulcer.	Streptococci and several unidentified bacilli. No pure cultures. 6 tubes inoculated; 2 contained streptococci.
C	Small groups of cocci, few bacilli, some of them diphtheroids, found in and on degenerating lining near edge of ulcer.	Streptococci, *Bacillus subtilis*, *Micrococcus tetragenus*, large Gram-positive bacilli, diphtheroid bacilli. 8 tubes inoculated. Streptococci pure in 1 and together with bacilli in another.
D	Few clumps of 15 to 20 small cocci on surface of ulcer.	Streptococci, yeast, *Staphylococcus albus*, Gram-positive and Gram-negative motile bacilli, Gram-positive non-motile bacilli. 14 tubes inoculated, 2 of which contained pieces of ulcer. In one of the latter streptococci were found in pure culture; in 1 other tube they occurred together with Gram-positive bacilli.
E	Large numbers of organisms singly and in clumps found on degenerating surface of lining of ulcer. These are mostly small cocci in long chains; many large bacilli and a few slender bacilli.	Streptococci were seen in cultures but could not be isolated; slender Gram-positive bacilli, staphylococci, spore-bearing Gram-positive bacilli, and a pleomorphic Gram-positive bacillus were isolated. 13 tubes inoculated, 2 of which contained pieces of ulcer. In the latter cocci in long chains were seen; 4 tubes were sterile.
G	Few organisms in and upon degenerating lining of ulcer. These are mainly large and small bacilli and a few cocci.	Streptococci, Gram-positive bacilli, motile Gram-negative bacilli, *Staphylococcus albus*, *Bacillus subtilis*, Gram-negative spore-bearing bacilli. 14 tubes inoculated, 2 of which contained pieces of ulcer. In 1 of the latter streptococci were found in almost pure culture and in the other there were a few chains of streptococci and Gram-negative motile bacilli. Of the remaining 12 tubes, 1 contained streptococci in pure culture, and 4 contained streptococci together with 1 or more other organisms.

TABLE I—*Concluded.*

Ulcer.	Types of organisms found in sections of ulcers.	Organisms recovered in cultures from ulcers.
J	Few bacilli on surface of ulcer. In a prolongation of the necrotic lining of the ulcer into the depth there are yeasts, scattered and in large clumps; small cocci, some of which are in chains; large cocci.	Streptococci, yeast, staphylococci, *Bacillus subtilis*, spore-bearing Gram-negative bacilli. 16 tubes inoculated, 2 with pieces of ulcer, which contained streptococci together with Gram-positive bacilli. In 3 of the remaining tubes streptococci were found in pure culture, in 4 together with other organisms, and 1 tube was sterile.
L	In and upon the degenerating lining are found masses of yeast but no bacteria.	Yeast, *Staphylococcus albus*, small Gram-positive bacillus. 12 tubes inoculated. None contained streptococci, but yeast and *Staphylococcus albus* were recovered from all.
M	In and upon the degenerating lining are many cocci, some in chains. There are as many bacilli present as cocci; some of the bacilli are large and in chains and some are small. Yeasts in tube-like stems are also found.	Streptococci, yeast, *Bacillus subtilis*, staphylococci, Gram-positive bacilli, large streptobacilli. 15 tubes inoculated, 2 with pieces of ulcer. The latter contained streptococci together with other organisms. Of the remainder, 2 contained streptococci in pure culture, 1 contained streptococci and bacilli, and 7 were sterile.
Gastrojejunal.	No bacteria demonstrable by Gram-Weigert method.	Streptococci, staphylococci, short Gram-positive bacillus. 11 tubes inoculated, 1 with piece of ulcer. Streptococci recovered in pure culture from 3 tubes and together with staphylococci in 2 tubes. 1 tube was sterile, 1 contained a short Gram-positive bacillus, and 4, including the tube inoculated with a piece of ulcer, contained staphylococci.

Plain Blood Agar.—Small discrete or fused colonies, gray by reflected light, and opaque and surrounded by a narrow green zone by transmitted light. No hemolysis.

From seven ulcers we recovered anhemolytic streptococci; streptococci were seen in cultures from the eighth but could not be isolated, and from the ninth no streptococci were recovered.

Yeasts were recovered from four ulcers. They were not identified, but grew luxuriantly aerobically on all the ordinary laboratory media. Morphologically slight variations in the strains were noted. They resembled morphologically the Saccharomyces described by Besson.[3]

Method of Inoculation.

Recently isolated cultures were injected in most of the experiments, the exact generation being given in the table, "first generation" meaning the original culture. The material for inoculation was prepared as follows:

1. A 24 hour growth of streptococci was obtained either in deep tubes of glucose-serum-bouillon or on slants of glucose-serum-agar. About 20 cc. of the growth in bouillon were centrifuged, washed twice with normal saline solution, and the resulting sediment was taken up in 2 to 5 cc. of normal saline solution. The 24 hour growth from one to four glucose-serum-agar slants was similarly washed with normal saline solution and the sediment resuspended in salt solution.

2. A milky emulsion of a 24 hour culture of yeast on glucose-serum-agar was made in several cc. of normal saline solution.

The entire amount of suspension prepared was used for intravenous injection into the ear vein and from 1 to 2 cc. for injection into a branch of the gastric artery. For the gastric artery injection the animals were etherized and a laparotomy was performed under aseptic precautions. The stomach was delivered and a branch of the gastric artery, usually on the anterior wall, was injected with a very fine needle and Record syringe. The part supplied always blanched during injection, but the color immediately returned on withdrawal of the needle. Very little hemorrhage followed and this was quickly controlled by compression. The wounds in every animal healed *per primam* and none developed peritonitis. Subsequent investigation was made through a laparotomy wound, opening the stomach widely and inspecting the entire mucosa directly. These gastric incisions also healed promptly and in several animals the stomach was opened a second time without apparent injury.

[3] Besson, A., Technique microbiologique et sérothérapique, Paris, 5th edition, 1911.

Animal Experiments.

Rabbits and cats were used in our experiments. Most of the rabbits were injected intravenously with streptococci, but in some the injections of either streptococci or yeast were made directly into a branch of the gastric artery. Streptococci were injected into a branch of the gastric artery in two cats and a yeast in the other two.

Table II gives in a condensed form the experiments and their results.[4]

Gastric Lesions in Rabbits Following Intravenous Injection of Streptococci.

Thirty rabbits were injected intravenously with streptococci. Gastric lesions developed in four (13.3 per cent).

Rabbit A III.—Killed 2 days after injection.

Stomach.—Over the anterior wall of the stomach near the cardia there are three pale, almost circular areas seen on the serosa. One of these is at the termination of a group of blood vessels springing from the lesser curvature. Beneath these areas are what appear to be defects in the mucosa, from 2 to 10 mm. in width. The edges are abrupt and irregular and one cannot tell whether the base of the defects is covered by the mucosa or not.

Microscopical Examination.—The defects extend almost through the mucosa, with slight inflammatory reaction about them. Gram-Weigert stain shows various types of bacilli and cocci along the entire surface of the mucosa and defect. The same organisms occur in the tissues just beneath the surface, but at no greater depth at the site of the defect than elsewhere. No predominance of cocci.

Rabbit C III.—Killed 1 day after injection.

Stomach.—The anterior wall of the cardiac region is bright red. The mucosa beneath this is also bright red. No defects found.

Microscopical Examination.—(Two blocks.) Section 1. From non-hemorrhagic area. Tissues are normal. There are no bacteria in the mucosa, but on the surface are found the large Gram-positive bacilli that occur on the surface of all stomach sections.

Section 2. From hemorrhagic area. There is an engorgement of the capillaries in the mucosa just beneath the surface, and slight extravasation. No bacteria in this area. At the other end of the section, which includes normal tissue, just beneath the surface of the mucosa, there is a very large clump of Gram-positive cocci, some of them in chains, but mostly occurring as diplococci or

[4] A number of experiments were performed with organisms recovered from other gastric ulcer cases, which we hope to embody in a later report.

TABLE II.

Stomach Lesions.

Ulcer.	Rabbit.	Days animal lived after injection.	Generation of streptococcus or yeast injected.	Intravenous injection.	Gastric artery injection.	After intravenous injection.	After gastric artery injection.	Heart lesions.	Cultures of urine, bile, and heart's blood.
			Streptococcus.						
A	I	1	2	+					
	II	4	2	+				Few hemorrhages.	Negative.
	III	2	2	+		Defect.		Hemorrhages.	
	IV	10	2	+					"
C	I	2	3	+				Endocardial hemorrhages.	
	II	3	3	+				Hemorrhages.	Streptococci from blood; urine and bile negative.*
	III	1 (Killed.)	4	+		Hemorrhages.		"	Streptococci and staphylococci in blood; urine and bile negative.†
	IV	3	4		+		Abscesses and small defects.	Gray streak in septum.	
	II 1	3	3 (From Rabbit C II.)	+				Pink and gray nodules in endocardium, epicardium, and valves.	Negative.
	II 2	6	3 (From Rabbit C II.)	+					"
D	I	5	1	+					Streptococci from heart's blood.
	II	3	1	+					Negative.
	III	2	3	+				Gray streak in septum.	Streptococci from blood and urine.‡

	IV	4	3	+				Gray streaks in heart.	
E	I	Died immediately.§	Original bouillon culture. Streptococci seen; not isolated.	+					
	II	1 (Died.)		+				Cocci found microscopically in blood vessels; no lesions.‖	
	III	1 "		+		Reddened; no hemorrhages.			Staphylococci and bacilli from heart's blood.**
G	I	2	1	+				Hemorrhages.	Negative.
	II	7	1	+					"
	III	7	1	+					"
	IV	2	2	+					"
	V	14	2	+					"
	VI	3	2	+		Hemorrhage and shallow defect.		Mottled appearance.	Streptococci from heart's blood.*
	VII	5	3		+		Defect.		Negative.
	VIII	4 (Died.)	3		+		Minute hemorrhages.		
J	I	7	1	+					"
	II	4	1	+					"
	III	7	4		+		Defect.		"
	IV	4	4	+					"
	V	7	4		+				
	VI	7	4	+				Hemorrhage in tricuspid valve.	"

* Gray streaks in skeletal muscles. † Hemorrhages in intestines and appendix. ‡ Tissues throughout body reddened. § Died with symptoms of anaphylactic shock. ‖ Hemorrhages in intestines. ** Tissues congested.

TABLE II—*Concluded.*

Ulcer.	Rabbit.	Days animal lived after injection.	Generation of streptococcus or yeast injected.	Intravenous injection.	Gastric artery injection.	After intravenous injection.	After gastric artery injection.	Heart lesions.	Cultures of urine, bile, and heart's blood.
			Streptococcus.						
M	I	7	1		+		Defect.		Negative.
	II	16	1		+				
	III	7	1 (Killed culture.)		+		Minute hemorrhages.		
	IV	2	1	+				Hemorrhages.	Streptococci from heart's blood.
	V	11	1	+					
Gastro-jejunal ulcer.	I	2	3	+					
	II	5	3	+					
Summary	38			30	8	4 (13.3 per cent.)	6 (75 per cent.)	16	
			Yeast.						
L	I	5	1	+					
	II	7	3		+				
	III	1	3	+		Hemorrhage.		Hemorrhages.	
	IV	7	3	+		"			
Summary	4			3	1	2 (66 per cent.)	0	1	

	Cat		Streptococcus.						
J	I	33	8		+		Small defect which healed.		
	II	33	8		+		Large defect which healed.		
Summary	2				2		2 (100 per cent.)		
			Yeast.						
L	I	12	4		+		Negative.		
M	I	13	2		+		"		
Summary	2				2		0		

as bacillary forms. There is no inflammatory reaction found around this area and only a slight, almost insignificant amount of extravasation of red blood cells.

Rabbit G IV.—Killed 2 days after injection.

Stomach.—On the anterior wall of the pyloric antrum, near the lesser curvature are two hemorrhagic areas in the mucosa, 2 by 4 mm. in size, and a few mm. from one another. There is a superficial defect over these areas and the mucous membrane around them for a distance of 2 mm. is slightly reddened.

Microscopical Examination—Serial sections of superficial defect. Gram-Weigert stain. A V-shaped defect, half way through the mucosa, in the midst of the hemorrhagic area. Very large bacilli are invading the defect from the surface. No streptococci. No bacteria in any of the blood vessels.

Rabbit G VI.—Animal became sick and was killed 3 days after injection.

Stomach.—On the mucosa on the anterior wall of the pyloric antrum there is a superficial hemorrhagic defect, 2 by 4 mm. The mucosa around it is reddened, and adherent to it is blood-stained mucus.

Microscopical Examination.—A considerable area of mucosa shows slight hemorrhagic infiltration, with hyaline thrombosis in a few of the superficial blood vessels and moderate degeneration of the superficial third of the mucosa. Just beneath the center of this area are several arteries and veins in the submucosa which show slight inflammation of their walls but no change in the lumen.

Serial Sections.—Gram-Weigert stain. No cocci in any of the vessels going to the mucosa. No bacteria except surface bacteria of various types.

Gastric Lesions in Rabbits Following the Injection of a Branch of the Gastric Artery with Streptococci.

In eight rabbits a branch of the gastric artery was injected with streptococci, according to the method described above. Gastric lesions developed in six (75 per cent).

Rabbit C IV.—Killed 3 days after injection.

The peritoneal cavity contains a small amount of fluid and clotted blood, and the omentum and portions of the stomach are infiltrated with blood. Omentum adherent over stomach. Serosa over injected area is pale, and shows beneath it a few pin-head, cream-colored areas, which look like miliary abscesses. Mucosa over these areas shows small hemorrhagic patches with slight defects in the mucosa, as well as miliary, cream-colored areas similar to the above.

Microscopical Examination.—There are several large hemorrhagic areas in the serosa and collections of leukocytes almost to the degree of abscess formation. There are about half a dozen depressions along the surface of the mucosa which are V-shaped, and about these depressions is a slight hemorrhagic infiltration with partial degeneration (?) of the structures. These areas reach half way through the mucosa. Over most of these depressions the surface epithelium is

intact but slightly degenerated and there is a slight fibrinous exudate adherent to the surface.

Gram-Weigert stain. Upon the mucosa over the hemorrhagic and purulent areas are a great number of bacteria, most of them large bacilli; no bacteria are found in the hemorrhagic and purulent areas in the mucosa. Bacteria are present in the serosa and correspond to those on the surface of the stomach. These are probably secondary invaders.

Rabbit G VII.—Killed 5 days after injection.

Stomach.—The branch of artery injected is identified by a small hemorrhagic spot at the site of injection. Distal to this the vessel seems empty. On the serous coat, in the portion directly supplied by the injected artery, an area, 1.5 by 3 cm., shows beneath the serosa a thin layer of thick, cream-colored pus. Beneath this is seen, from the mucosal side, an irregular, punched out defect, 1 by 0.5 cm., with raised, edematous edges, and an area of edema around it, 2 cm. in width. The base of the defect is formed by a very thin, transparent portion of the wall of the stomach. About 2 cm. from the defect is a hemorrhage in the mucosa, 2 mm. wide. The remainder of the stomach is normal.

Microscopical Examination.—The base of the defect shows degeneration and necrosis almost as far as the serosa. The edge of the defect is fairly sharp and shows a similar condition of degeneration.

Gram-Weigert stain. In the depth of the defect, just beneath the serosa and reaching for a short distance into the muscularis, are found solid clumps of streptococci filling the capillaries and small blood vessels. Between these, scattered throughout the tissues, are diffuse collections of streptococci. On and immediately beneath the necrotic surface of the defect are large clumps of bacilli. Individual bacilli also lie scattered between the clumps.

On cross section of a portion of the stomach with arteries near the defect, one of the arteries is seen to be filled with an organizing thrombus; the other arteries are normal. There are no bacteria in the thrombus.

Rabbit G VIII.—Died 4 days after injection.

Stomach.—About one-third filled with food. Delicate adhesions between the cardiac half of stomach and surrounding viscera. Over the cardiac half of the stomach, especially near the greater curvature, the mucosa shows numerous minute hemorrhages. No defects.

Microscopical examination was not made.

Rabbit J III.—Killed 7 days after injection.

Operation.—Attempt to make injection into a branch of the gastric artery near the cardiac end of the stomach on the anterior wall failed. Injection was made into another blood vessel on the anterior wall of the stomach, though it could not be determined whether it was a vein or an artery. No blanching of the portion of the stomach injected. Previous attempt at injection resulted in a large hematoma at the lesser curvature of the stomach. Two large branches of the gastric artery on the posterior wall of the stomach were injured in delivery of the stomach. Animal returned to cage in good condition.

Stomach.—Hematoma along the lesser curvature. About the vessel on the anterior wall which was injected there is an area about 1 by 0.5 cm. showing hemorrhages and a small amount of thick pus beneath the serosa. On the posterior wall of the fundus near the greater curvature, corresponding to the area supplied by the injured arteries, the stomach is adherent in two places to one of the lobes of the liver. The mucosa of the stomach immediately beneath these adherent areas shows an irregular defect 0.5 cm. in width which extends half way down through the gastric wall. The base of the defect is reddened; the edge is edematous and slightly reddened. Beneath the hemorrhagic areas, on the anterior wall of the stomach where the injection was made, there are no changes in the mucosa. The stomach is otherwise negative.

Microscopical Examination.—Defect on posterior wall. Superficial defect half way through the mucosa with degenerative changes, slight hemorrhages, etc. One large vein in the submucosa shows beginning mural thrombosis. No streptococci or other bacteria in the depth of the defect. Various types of bacteria present on the surface of the normal portion of the stomach and on the surface of the defect. Adhesions over the serosa contain no bacteria. Gastric arteries supplying the area of the defect are normal.

Gram-Weigert stain. Usual types of bacteria on surface of defect and neighboring normal mucosa, none in tissues except a few surface type organisms in the hemorrhagic area of the serosa.

The serosa of lesions on the anterior wall shows edema and slight hemorrhagic and leukocytic infiltration. Vessels and mucosa are normal. Gram-Weigert stain. Usual surface bacteria are present, but no organisms are found in the tissues.

Rabbit M I.—Killed 7 days after injection.

Stomach.—On the serous surface of the anterior wall of the fundus, in the center of the region supplied by the injected artery, there is a creamy yellow area, 1 by 2 cm., where the omentum is adherent. There are other smaller areas of thickening of serosa. Beneath the large area, practically the entire thickness of the stomach wall from the mucosa outward is necrotic, forming a defect with hemorrhagic edge and necrotic base. The branch of artery injected is in good condition.

Microscopical Examination.—There is a defect of the mucosa. Beneath the defect the gastric wall shows large masses of pus cells, edema, congestion, and degeneration of tissues, amounting almost to necrosis in places. No thrombi in veins or arteries.

Gram-Weigert stain. There are masses of cocci in the inflamed and degenerating area and similar masses in the overhanging edge of the defect apparently lying in the capillaries or lymphatics.

Rabbit M III.—Killed 7 days after injection with killed culture of streptococci.

Stomach.—Minute scar at the point of injection of the artery. Mucosa of fundus, near the greater curvature, shows a few minute hemorrhages but no defect over them.

Microscopical Examination.—Congestion of superficial capillaries. No hemorrhages. Gram-Weigert stain. No cocci, or other bacteria, except a few usual types on the surface.

Gastric Lesions in Cats Following the Injection of a Branch of the Gastric Artery with Streptococci.

In two cats a branch of the gastric artery was injected with streptococci. Both animals developed defects in the gastric mucosa, which soon began to heal and were observed to have healed completely in 33 days.

Cat J I.—Cat well 9 days after injection.

Stomach Operation.—Explored under ether anesthesia. Few adhesions found between the anterior wall of the stomach and the omentum, and between the posterior wall and the posterior wall of the lesser peritoneal sac. Along the lesser curvature there is an indurated area about 1 cm. wide in the region supplied by the injured artery. Beneath this is seen a punched out area in the mucosa that may be either a shallow erosion or healing defect. Stomach otherwise negative. The incision in the stomach, which was parallel to the greater curvature and 1 cm. from it on the anterior wall, was closed with a double row of sutures. 33 days after injection the abdomen was opened under ether anesthesia. No sign of defect found on serous or mucous coats of stomach. Animal killed.

Cat J II.—18 days after injection. Cat has remained entirely well and well nourished.

Operation.—Performed under ether anesthesia. Stomach free and nowhere adherent. At the lesser curvature, where the injected artery enters the stomach, there is an indurated area involving the entire wall of the stomach, 1.5 cm. wide and about the normal stomach wall in thickness. The stomach was opened on the anterior wall parallel to the greater curvature for a distance of 4 cm., 1.5 cm. from the greater curvature and the same distance from the indurated area. Beneath this indurated area is a shallow, irregularly round defect in the mucosa with indurated margins. The edge of the defect is formed by a bright red line, apparently of granulations, as though it were healing. Stomach closed. Animal returned to cage in good condition. 33 days after the injection the cat was again etherized. Slight puckered scar of serosa over the site of the previous defect. Mucosa at corresponding point shows slightly puckered area, entirely healed. Animal killed.

Microscopical Examination.—The mucosa is completely regenerated over the site of the ulcer. It is made up of glands which are slightly atypical. A slight defect in the submucosa has also been repaired. Gram-Weigert stain shows no bacteria.

Gastric Lesions in Rabbits Following Injection with Yeast.

Three rabbits were injected intravenously. Two of these animals developed minute hemorrhages in the gastric mucosa.

Rabbit L III.—Died 12 hours after injection.

Stomach.—Many minute hemorrhages in mucosa.

Microscopical Examination.—Negative; no yeast.

Rabbit L IV.—Killed 7 days after injection.

Stomach.—Few minute hemorrhages in mucosa, not examined microscopically.

In addition a branch of the gastric artery was injected in one rabbit (L II). The animal was killed on the 7th day and showed no lesions at autopsy.

Gastric Lesions in Cats Following Injection of Yeast into a Branch of the Gastric Artery.

Two cats (L I, M I) were injected. The animals died on the 12th and 13th days respectively, and no lesions were found at autopsy.

Summary of Cardiac Lesions.

Of the thirty rabbits injected intravenously with streptococci, fourteen (47 per cent) developed cardiac lesions. In two, minute vegetations containing streptococci were present. The remainder showed either petechial hemorrhages or the gross appearance of myocarditis, and practically all of these hearts contained microscopical lesions of the Bracht and Wachter type.[5]

Of the eight rabbits in which a branch of the gastric artery was injected two (25 per cent) developed minute hemorrhages in the endocardium.

Of the three rabbits injected intravenously with yeast one showed hemorrhages in the endocardium.

DISCUSSION.

As a result of the above studies it is impossible to decide definitely whether or not the gastric lesions produced by the injection of streptococci are to be considered ulcers. The superficiality of the rabbit lesions following the injection by the intravenous route as well as the entire absence of inflammatory reaction in the deeper gastric

[5] Thalhimer, W., and Rothschild, M. A., *Jour. Exper. Med.*, 1914, xix, 429.

tissues leads us to the belief that these defects are certainly not analogous to the chronic gastric ulcer seen in the human stomach. The promptness of healing of the embolic lesions in the cats tends to strengthen this conclusion. This point will be discussed more fully below.

Apparently striking results were obtained in Cats J I and J II, where the injection of streptococci into a branch of the gastric artery resulted in the formation of shallow, indurated defects that might be considered ulcers. However, these defects were found to be healing on the 10th and 18th days respectively, and were completely healed on the 33rd day.

It has been demonstrated by Bolton,[6] Wilensky and Geist,[7] and others that defects produced mechanically in the gastric mucosa and muscularis of cats heal in 2 to 4 weeks. In the defects produced in the rabbits by injection into a branch of the gastric artery streptococci were found in great numbers in the tissue about the lesions. By analogy it may be assumed, even in the absence of microscopic proof, that streptococci were also present at some time in the lesions in the cats. The embolic lesions in the stomachs of the cats in our series healed spontaneously within approximately the same length of time as those mechanically produced. It is evident, therefore, that in these instances the injected streptococci failed to retard the process of healing.

The constant presence of an anhemolytic streptococcus in human gastric ulcers might be adduced as an argument in favor of the part played by this organism as the cause of the chronicity of the lesion. If streptococci could be demonstrated in considerable numbers in the depth of human gastric ulcer this conception would gain a firmer basis. Rosenow[1] has found streptococci in all the coats of the stomach, some of the organisms even lying just beneath the serosa. No mention is made of the number of organisms in this position. In our series of ulcers, however, despite the examination of many sections, the streptococci were found only on or beneath the surface of the defect.

[6] Bolton, C., *Jour. Path. and Bacteriol.*, 1915, xx, 133.

[7] Wilensky, A. O., and Geist, S. H., *Jour. Am. Med. Assn.*, 1916, lxvi, 1382.

The case of gastrojejunal ulcer described above seems of importance in this connection. This ulcer might readily be considered as due primarily to purely mechanical factors. Streptococci were recovered culturally from emulsified pieces of tissue. Although the histological picture was identical with that of the other ulcers in the series the most careful search failed to show the presence of streptococci on the surface of the lesion or in the tissues about it. One must consider the possibility that in this case the streptococci were directly deposited upon a preexisting mechanical defect and were not the essential causative factor in the formation of the ulcer.[8]

A possible explanation of the occurrence of streptococci in gastric ulcers may be found in some observations of Cushing and Livingood.[9] These investigators found that whereas there is a more or less definite bacterial flora of the ileum and large intestine, this is not true of the upper portion of the alimentary tract. The stomach and the duodenum and at times also the upper jejunum become free or practically free of bacteria after a certain period of fasting, whereas during and shortly after a meal great numbers of organisms of different types are found. These are greatly reduced in number when sterile food alone is taken. Cushing and Livingood, therefore, concluded that the normal stomach has no definite bacterial flora but that this is dependent upon the bacteria taken with the food.

[8] Since this article was completed we have studied another gastrojejunal ulcer that had the gross and microscopic appearance of a chronic gastric ulcer. No bacteria were found with the Gram-Weigert stain either upon the surface of the lesion or in the underlying tissues. Anhemolytic streptococci were isolated from several tubes of glucose-serum-agar inoculated with the emulsified ulcer. Other bacteria similar to those described in the body of the paper were also recovered.

Six rabbits were injected intravenously with the second generation of the streptococcus and were killed at periods varying from 2 to 10 days. At autopsy there were small hemorrhages in the duodenal mucosa of one rabbit, hemorrhages in the tricuspid valve in two others, and minute tricuspid vegetations in a fourth. The structure of these vegetations on microscopical examination as well as the absence of streptococci in smears and sections indicates that the injected organisms were not the cause of the lesion. No other lesions were found in these four animals, and in the remaining two the autopsy was entirely negative. No streptococci were found either culturally or microscopically in the hemorrhagic area in the duodenum.

[9] Cushing, H., and Livingood, L. E., *Johns Hopkins Hosp. Rep.*, 1900, ix, 543.

Cushing and Livingood showed that neither the acidity nor the digestive ferments of the gastric secretion have marked lethal effect on the bacteria in the stomach. They believe that the stomach becomes aseptic chiefly through the mechanical removal of organisms in the passage of the ingesta, and that bacteria cannot adhere to normal gastric mucosa nor do they enter or remain in the glandular crypts. Whether bacteria can adhere to the surface or penetrate into the depths of defects in the mucosa remains to be determined. These writers further state that there were two organisms from which the stomach freed itself with difficulty, and which were found in a number of examinations many hours after the intake of food. These organisms were a minute Gram-positive diplococcus and yeast. The same forms were found almost constantly in our series of gastric ulcers both in sections and in cultures.

In the light of the work of Cushing and Livingood one must bear in mind the possibility that these organisms which are commonly found in the stomach and duodenum might remain upon or immediately beneath the degenerating lining of the ulcer because of favorable mechanical or structural conditions.

The nature of both the experimental gastric and cardiac lesions indicates that the streptococci recovered from the ulcers are of a low grade of pathogenicity, such as is usually found in the same type of organism isolated from other sources. Whether, having established themselves on the surface of a gastric ulcer in man, they are innocuous or manifest a low grade of pathogenicity tending to retard healing requires further investigation.

In addition to the streptococcus other organisms were invariably recovered from the inoculated tubes. No attempt was made to identify these strains culturally. The same types were usually identified microscopically in sections, proving that the isolated organisms other than streptococci were not accidental contaminations. Furthermore, these bacteria were quite as numerous in the sections as streptococci, and penetrated to the same depth in the ulcer. The types most frequently encountered were a yeast and a thick Gram-positive bacillus. Some of the isolated strains of yeast proved pathogenic for rabbits on intravenous inoculation. Our data are insufficient to warrant an expression of opinion as to the significance of these organisms.

CONCLUSIONS.

It must be assumed that some cause is operative in certain cases preventing the healing of defects in the gastric mucosa and is inoperative in others. Even though anhemolytic streptococci are present in practically all gastric ulcers, we cannot convince ourselves that these organisms have been proven as yet to be the factor which either initiates the ulceration or prevents healing. Nevertheless, the constant presence of streptococci in this type of lesion is a suggestive fact and further experiments to determine their significance are being undertaken.

We wish to express our thanks to Dr. A. A. Berg, at whose suggestion this investigation was undertaken. We are further indebted to him for the material studied, for the privilege of reporting abstracts from his clinical records, and for other courtesies extended to us during the progress of this work.

INDEX TO AUTHORS.

INDEX TO SUBJECTS.

38 1/2

FIG. 1.

(Larkin and Levy: Syphilitic Aortitis.)

FIG. 2.

(Larkin and Levy: Syphilitic Aortitis.)

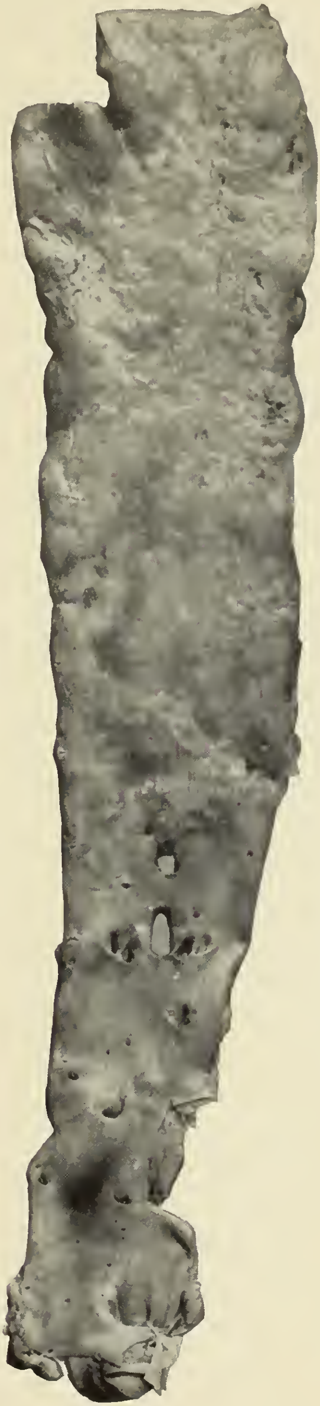


Fig. 3

(Larkin and Levy: Syphilitic Aortitis.)

FIG. 4.

(Larkin and Levy: Syphilitic Aortitis.)

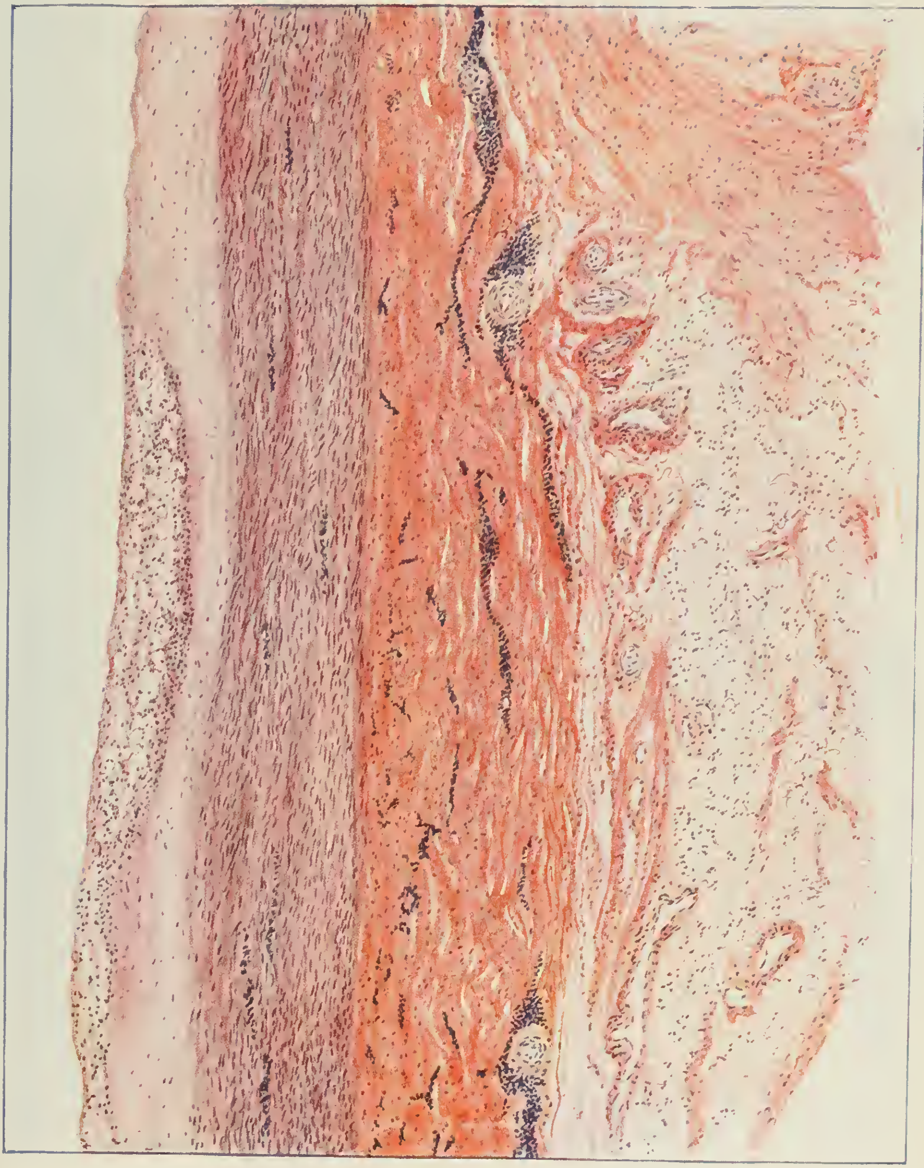


Fig. 5.

(Larkin and Levy: Syphilitic Aortitis.)

FIG. 6.

(Larkin and Levy: Syphilitic Aortitis.)

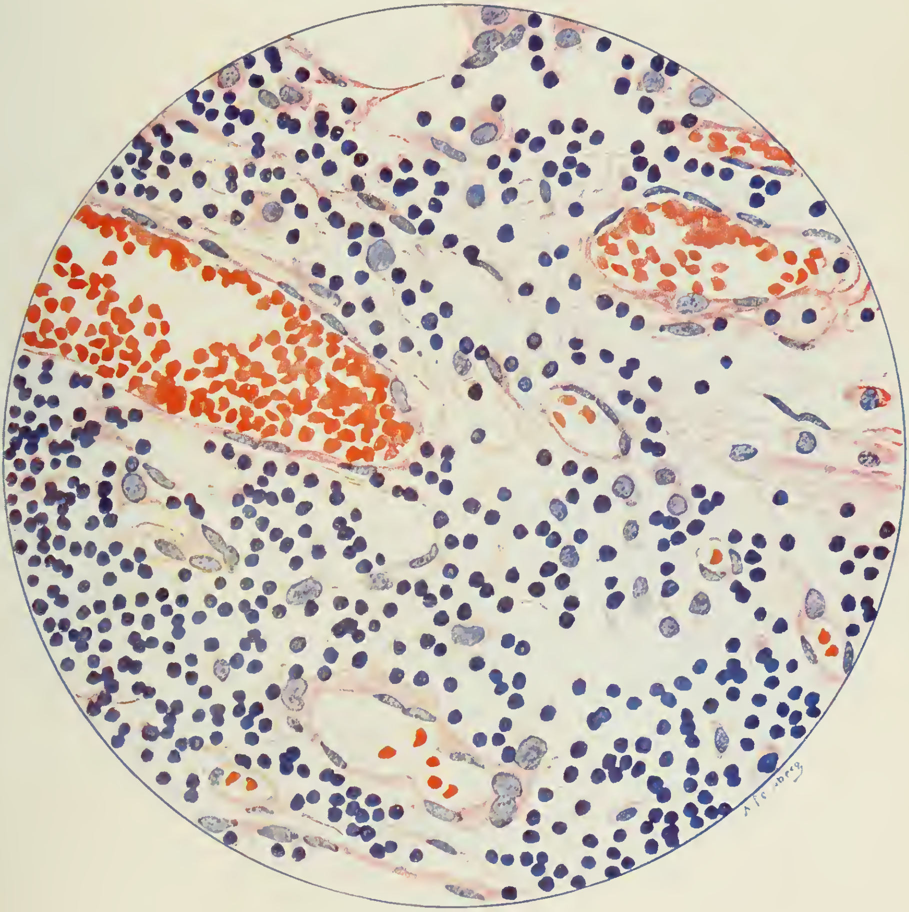
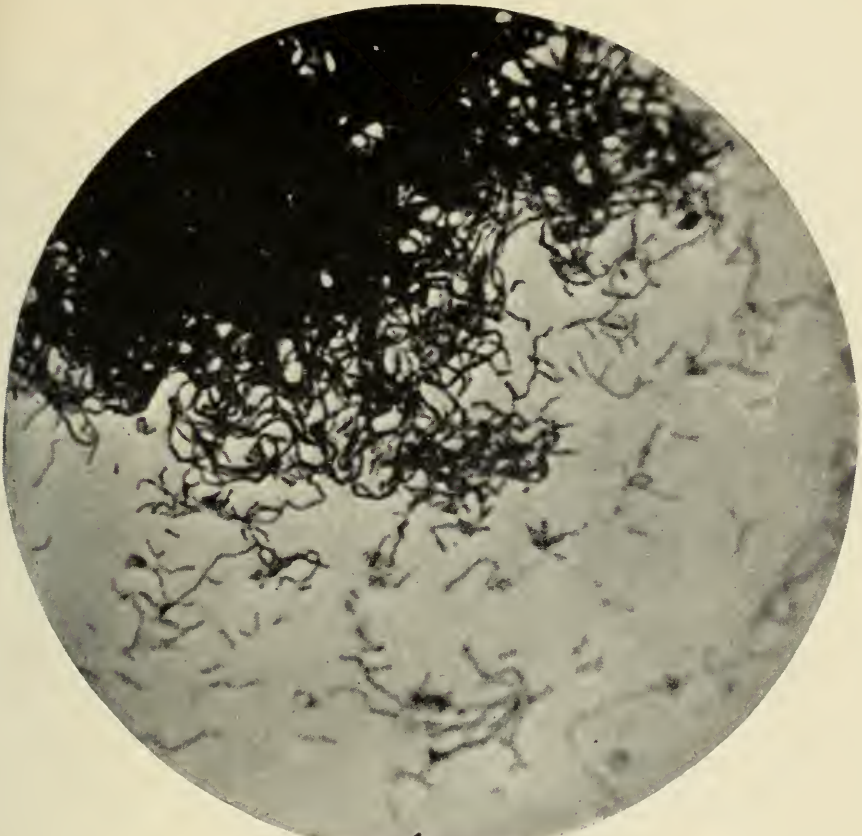


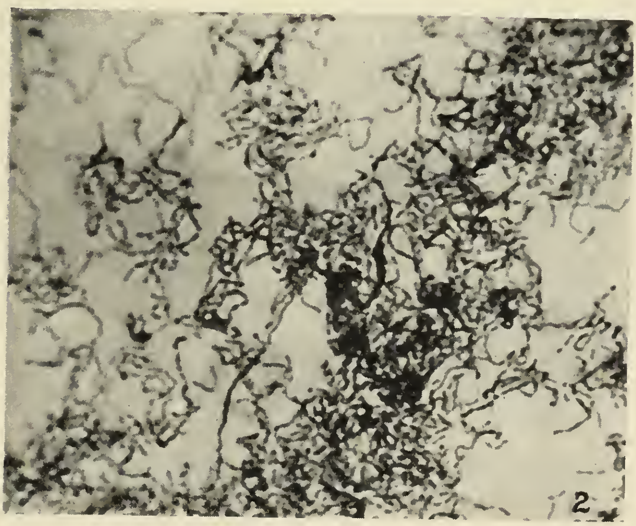
FIG. 7.

(Larkin and Levy: Syphilitic Aortitis.)

60'

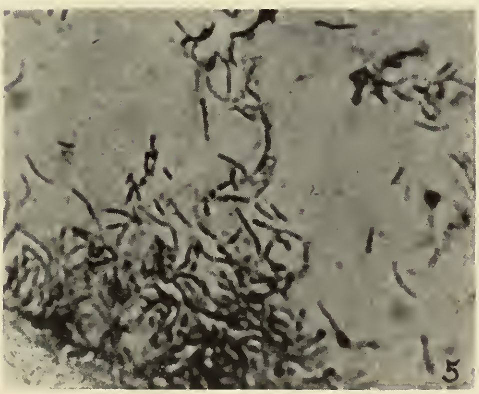


1



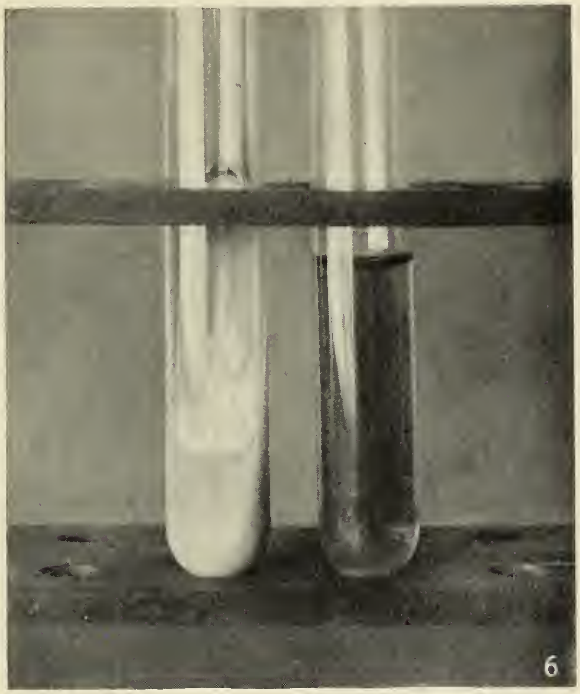
2

(Blake: Etiology of Rat-Bite Fever.)



(Blake: Etiology of Rat-Bite Fever.)

60[3]

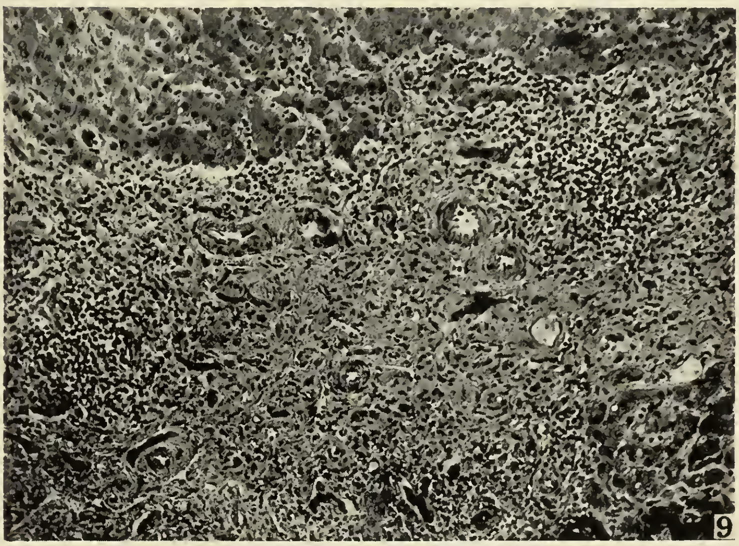


6

7

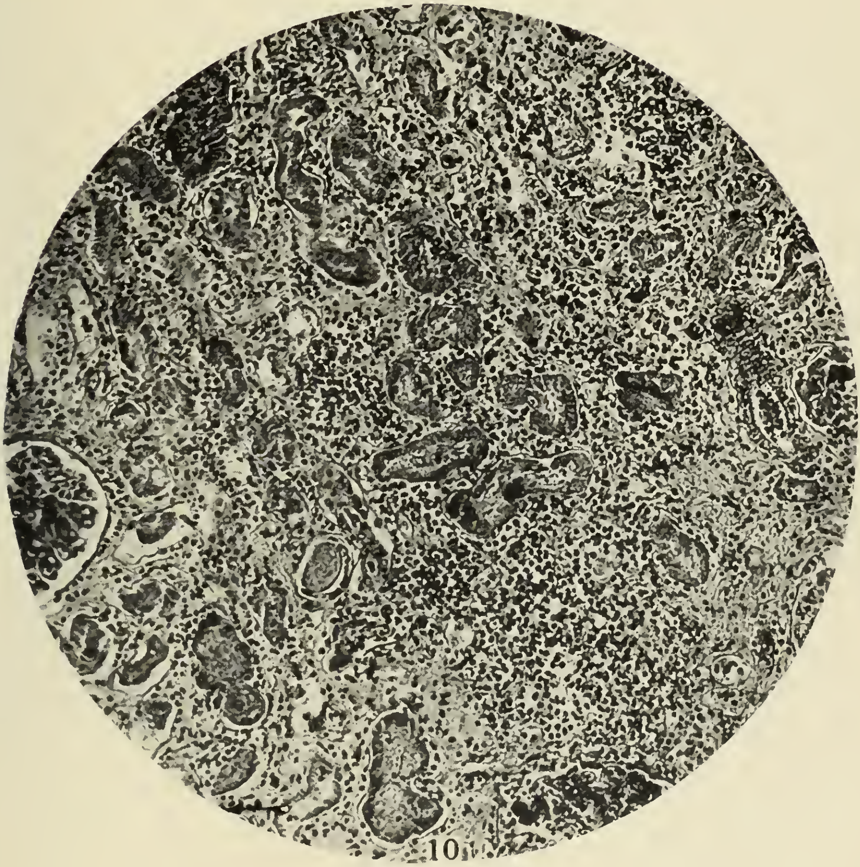
(Blake: Etiology of Rat-Bite Fever.

8



9

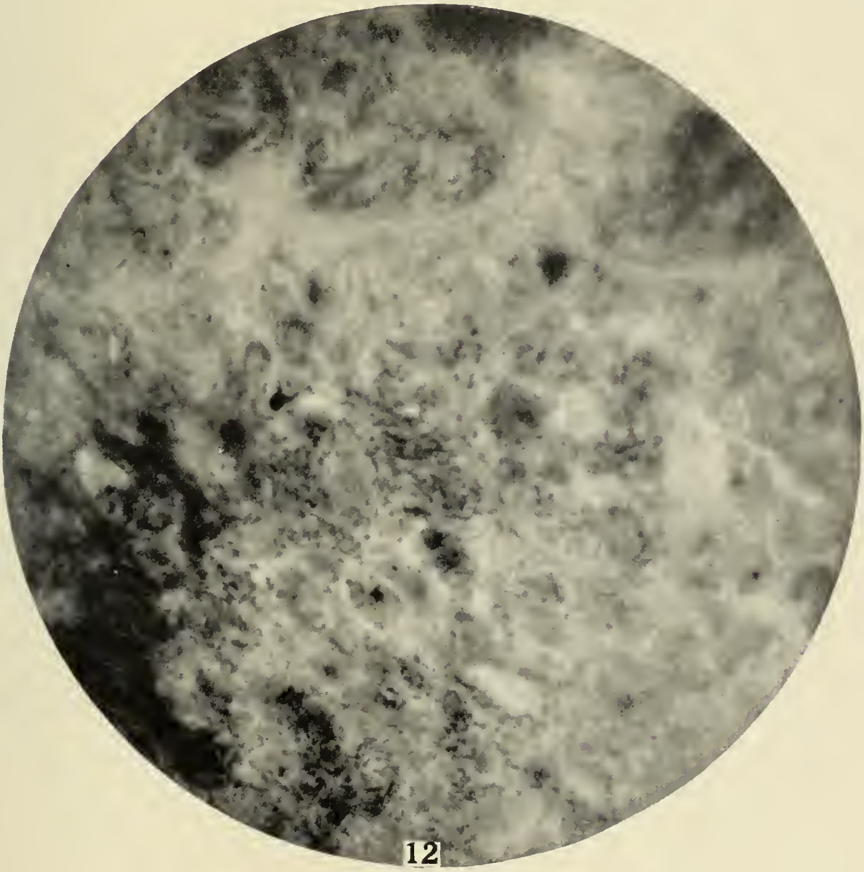
(Blake: Etiology of Rat-Bite Fever.)



(Blake: Etiology of Rat-Bite Fever.)

(Blake: Etiology of Rat-Bite Fever.)

60[7]



12

(Blake: Etiology of Rat-Bite Fever.)

FIG. 1.

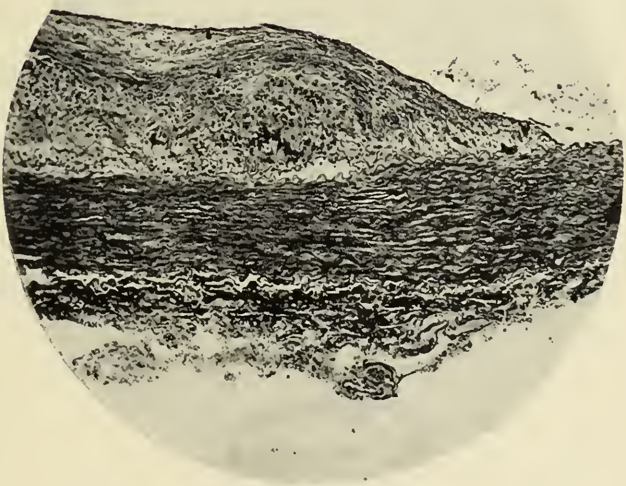


FIG. 2.

(Bailey: Atheroma Produced by Cholesterol Feeding.

FIG. 3.

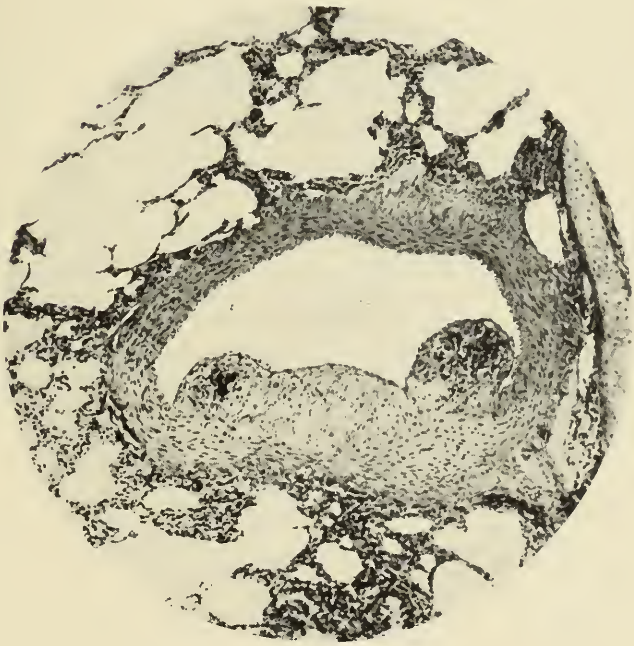


FIG. 4.

(Bailey: Atheroma Produced by Cholesterol Feeding.)

74

Fig. 5.

Fig. 6.

(Bailey: Atheroma Produced by Cholesterol Feeding.)

FIG. 7.

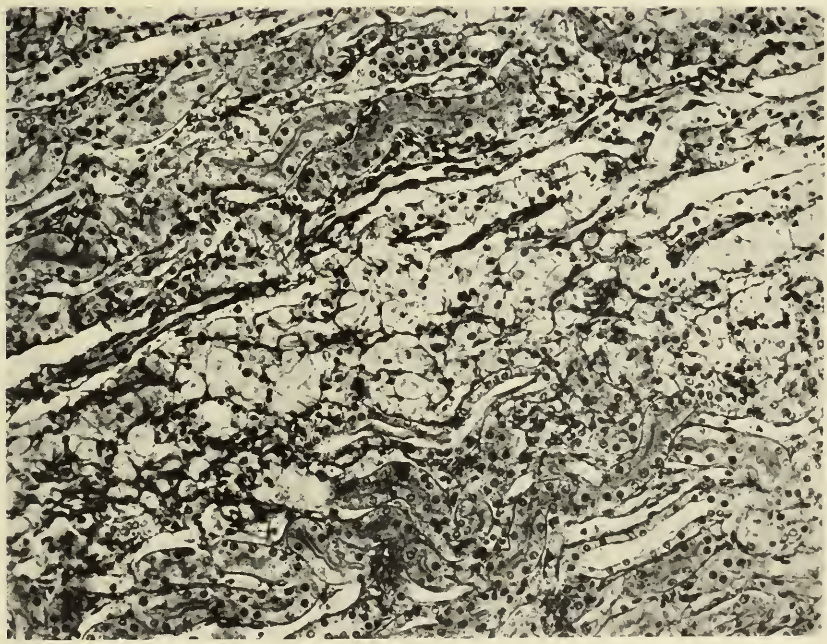


FIG. 8.

(Bailey: Atheroma Produced by Cholesterol Feeding.)

FIG. 9.

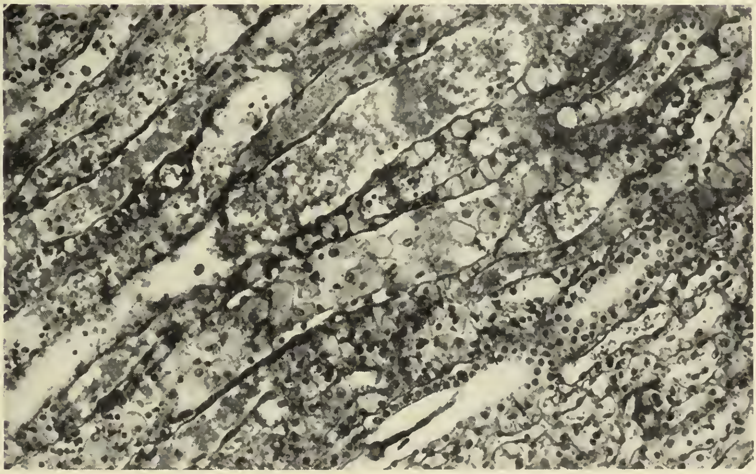


FIG. 10.

(Bailey: Atheroma Produced by Cholesterol Feeding.)

170'

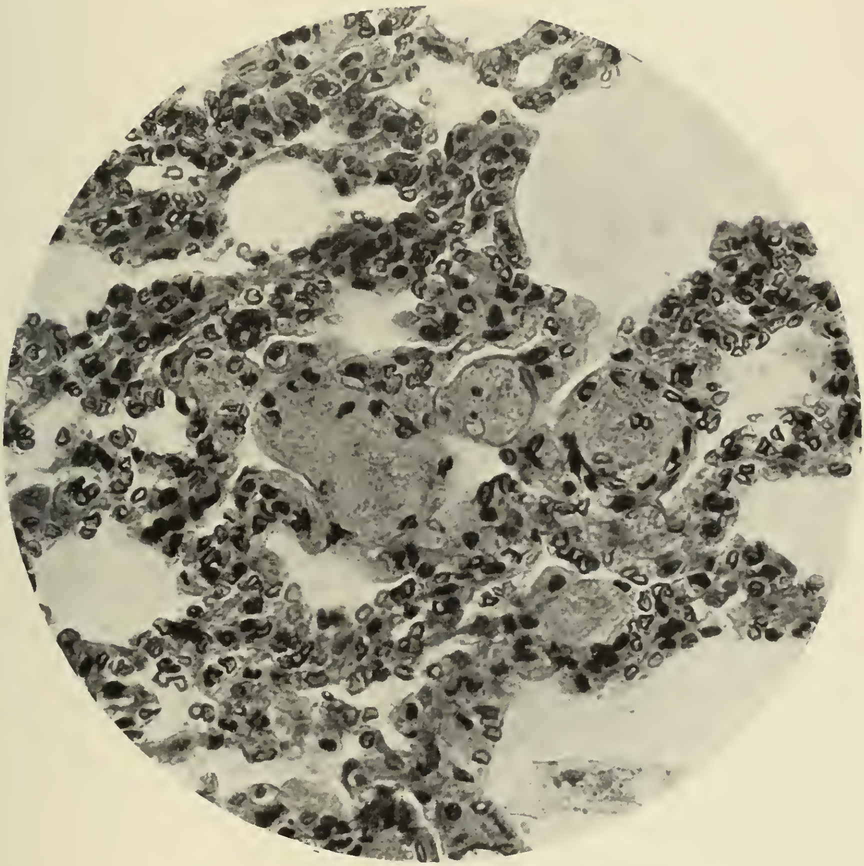


FIG. 1.

(Karsner: Atmospheres Rich in Oxygen.)

THE JOURNAL OF EXPERIMENTAL MEDICINE VOL. XXIII. PLATE 21.

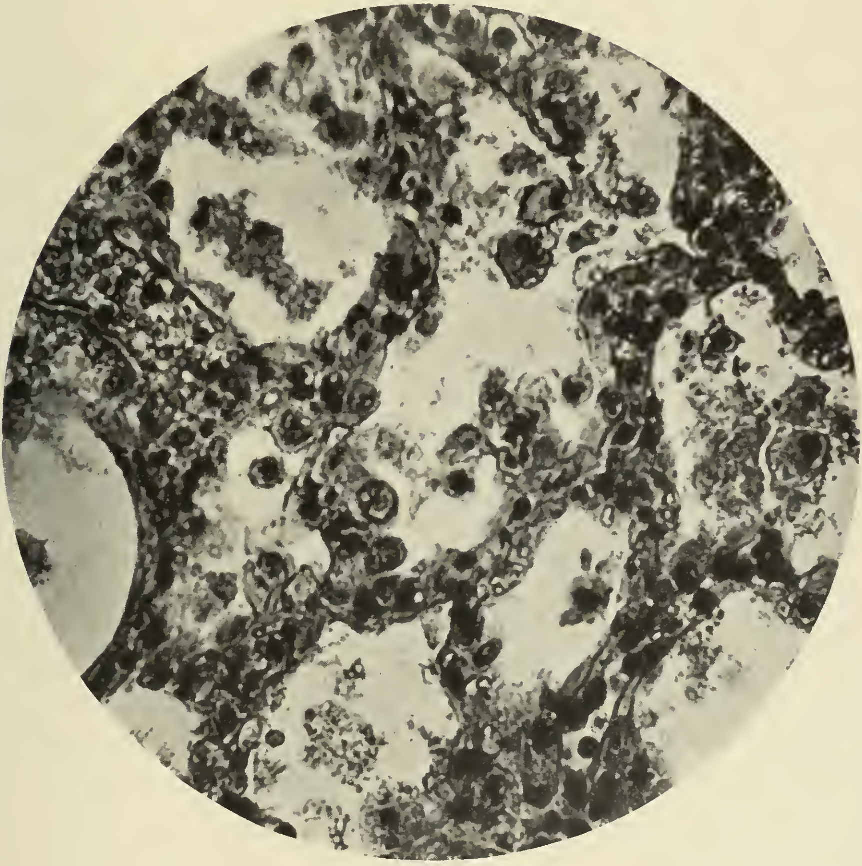


FIG. 2.

(Karsner: Atmospheres Rich in Oxygen.)

170

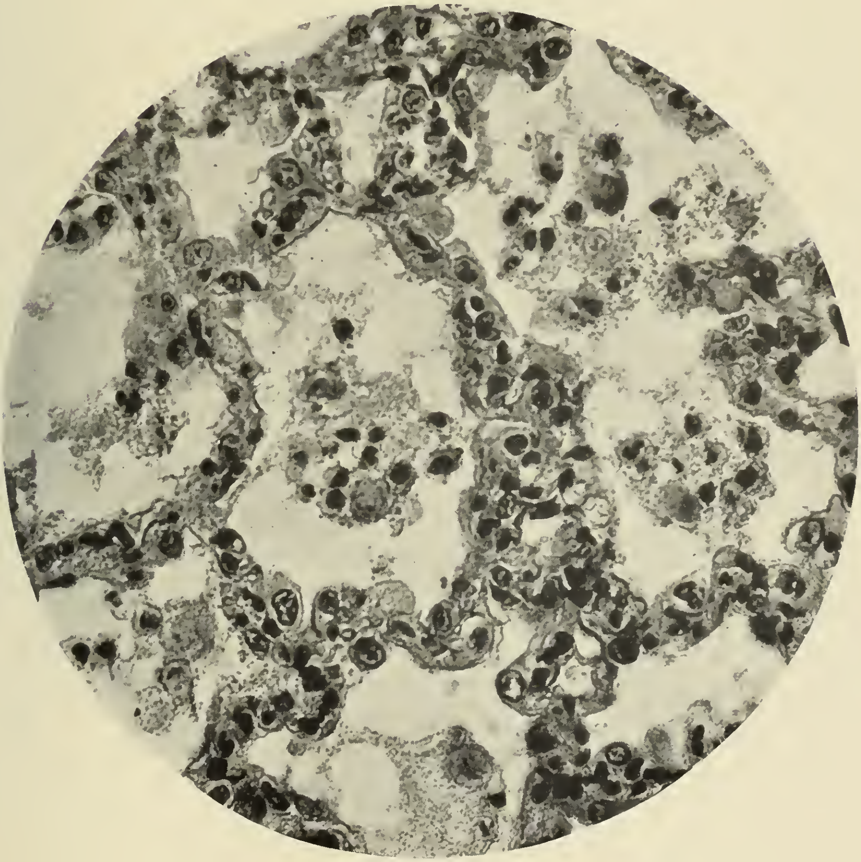


Fig. 3.

(Karsner: Atmospheres Rich in Oxygen.)

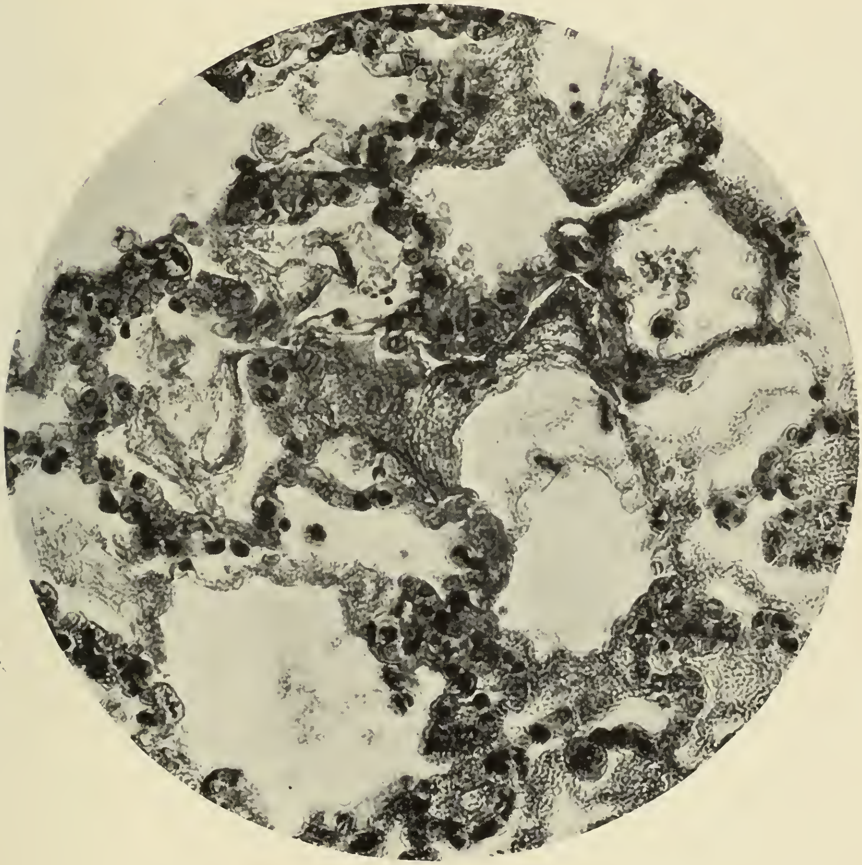


FIG. 4.

(Karsner: Atmospheres Rich in Oxygen.)

186ª

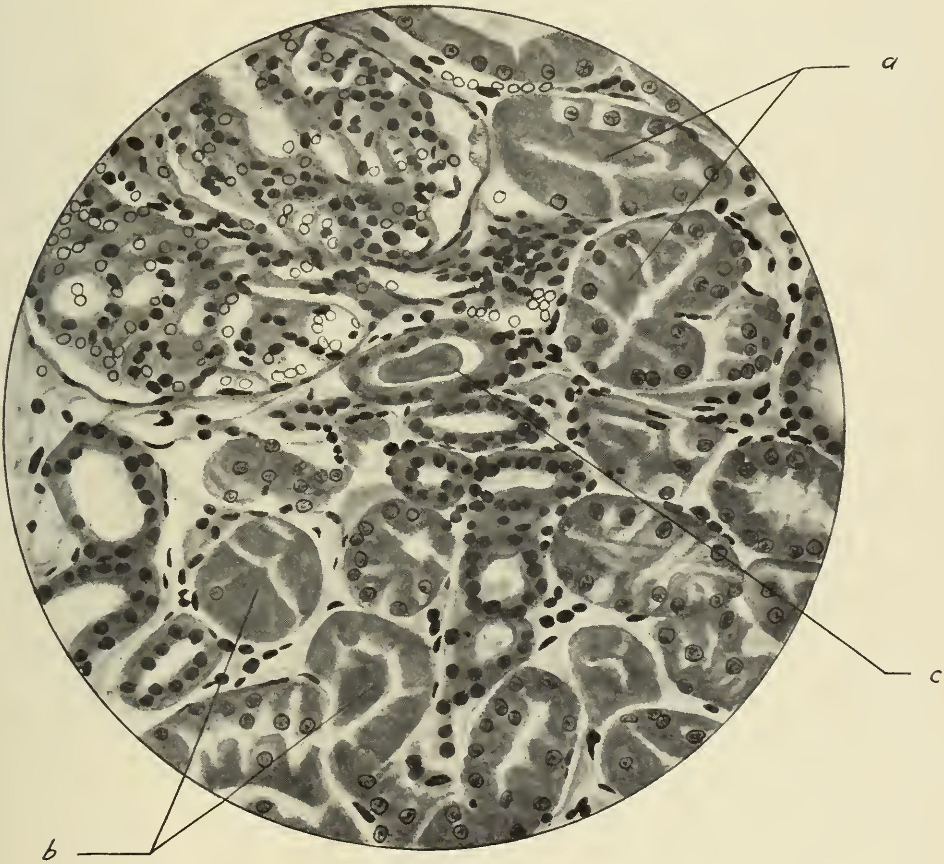


FIG. 1.

(MacNider: Inhibition of Toxicity of Uranium.)

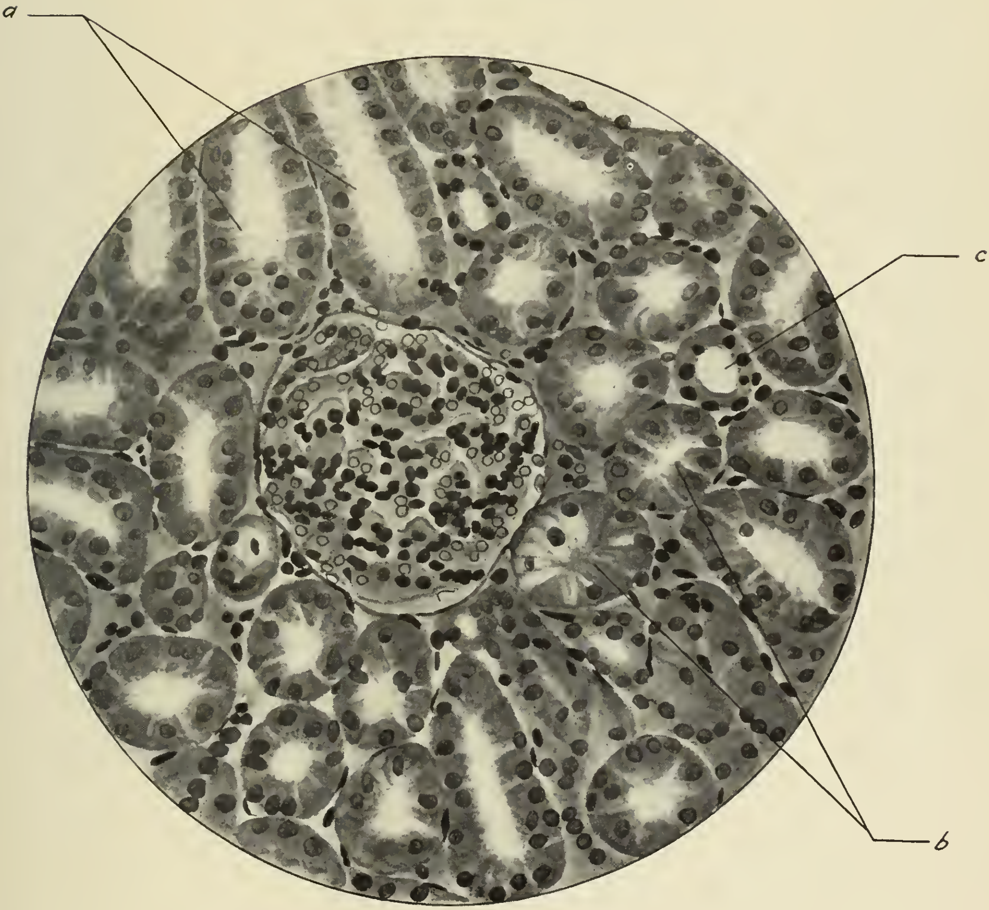


Fig. 2.

(MacNider: Inhibition of Toxicity of Uranium.)

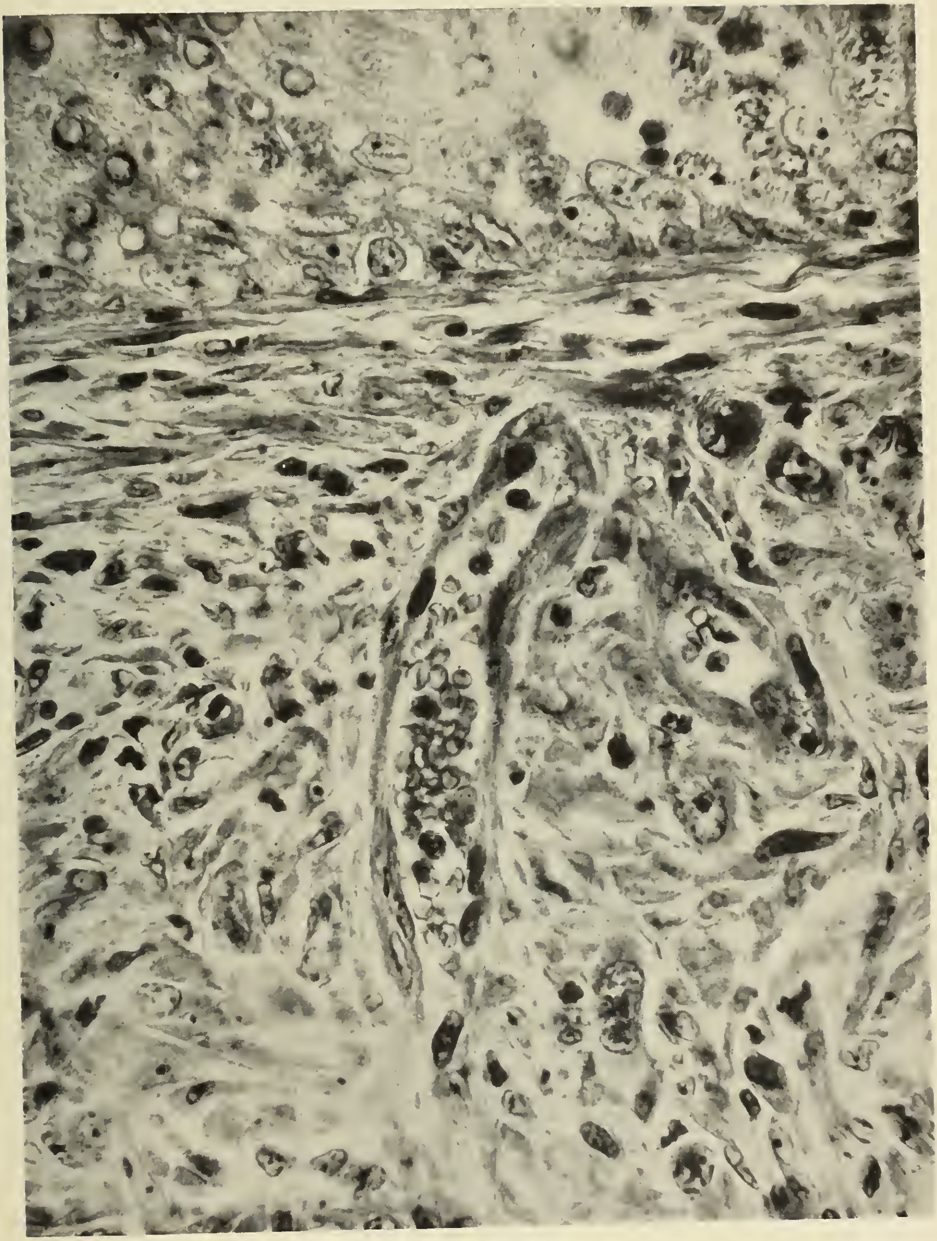


Fig. 1.

(Woglom: Flexner-Jobling Rat Carcinoma.)

Fig. 1.

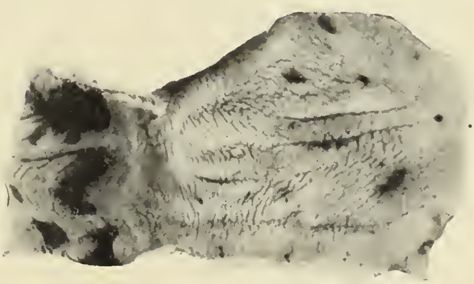


Fig. 2.

(Mann: Gastric Ulcers.)

Fig. 3.

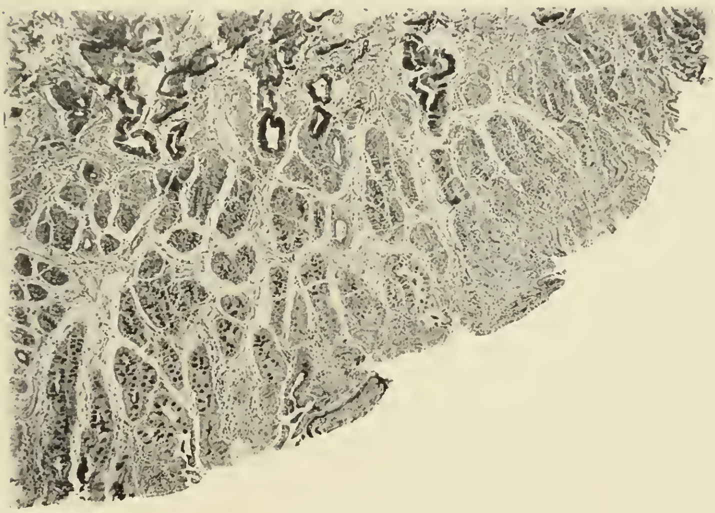
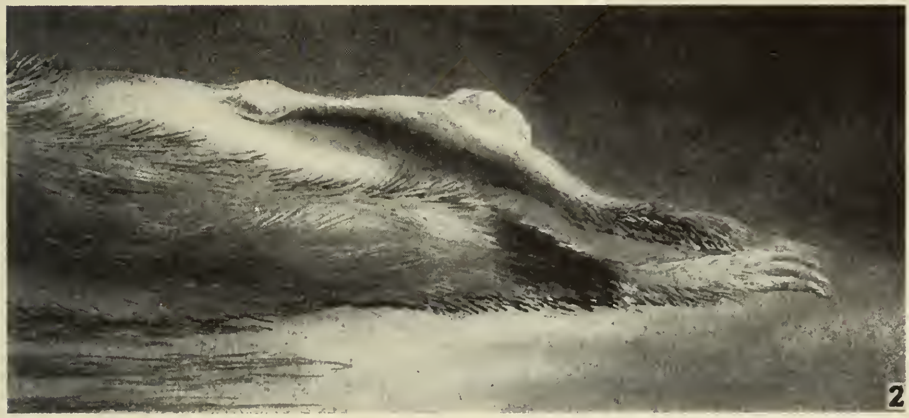


Fig. 4.

(Mann: Gastric Ulcers.)

1



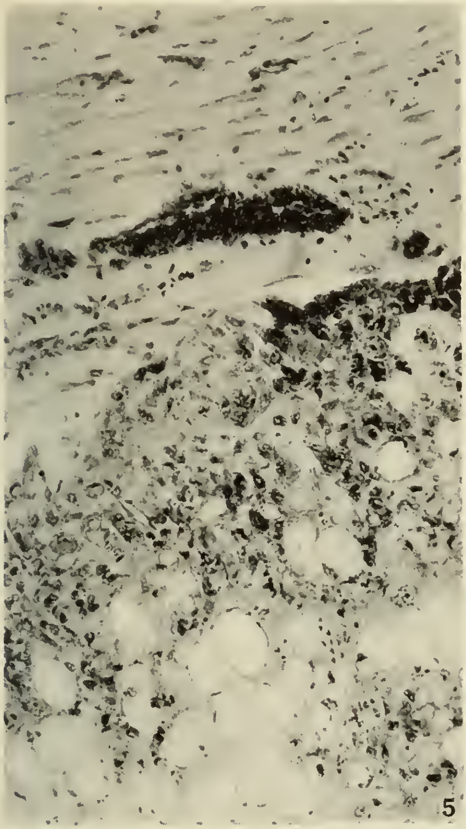
2

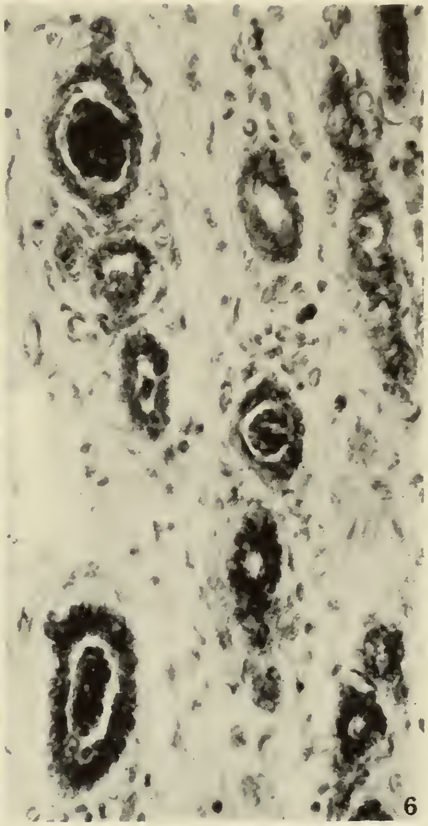
3

(Jones: Transplantable Carcinoma of Guinea Pig.)

218[2]

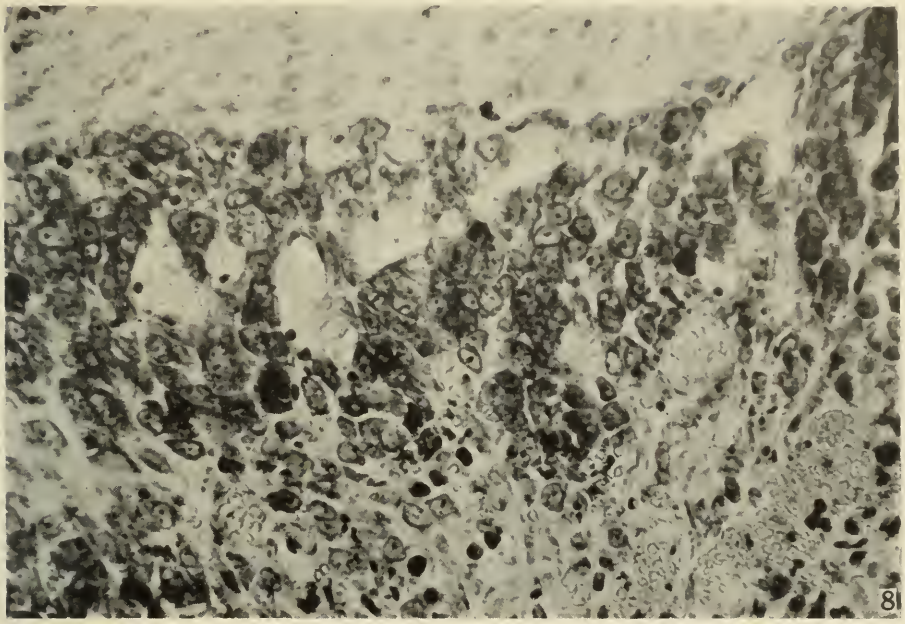
(Jones: Transplantable Carcinoma of Guinea Pig.)

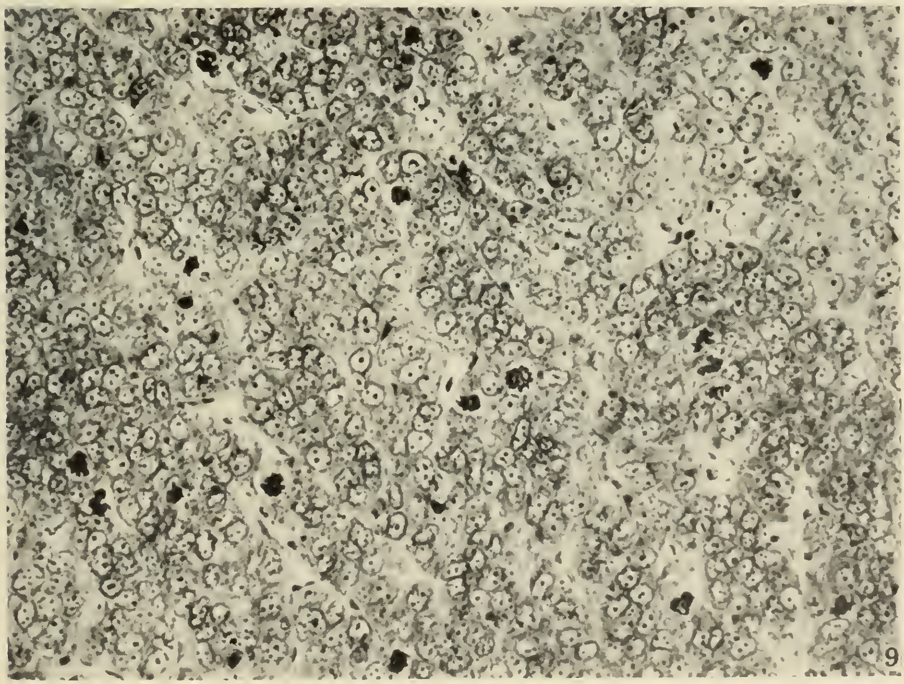




(Jones: Transplantable Carcinoma of Guinea Pig.)

218





(Jones: Transplantable Carcinoma of Guinea Pig.)

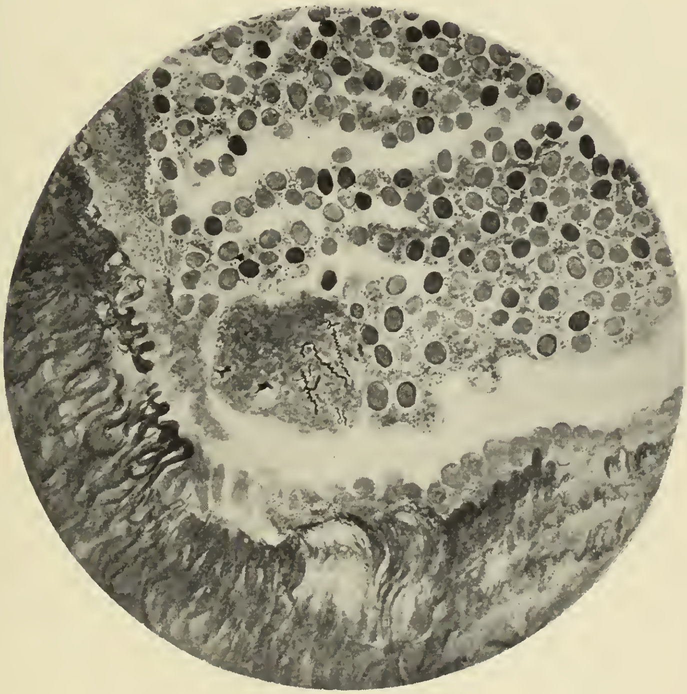


FIG. 1.

(Futaki, Takaki, Taniguchi, and Osumi: Cause of Rat-Bite Fever.)

Fig. 1.

Fig. 2.

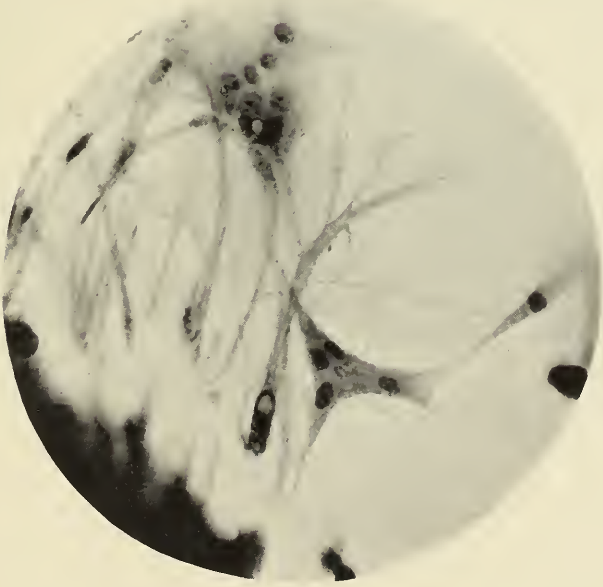


Fig. 3.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

264[2]

Fig. 4.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

 2 4 3

Fig. 5.
(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

THE JOURNAL OF EXPERIMENTAL MEDICINE VOL. XXIII. PLATE 37.

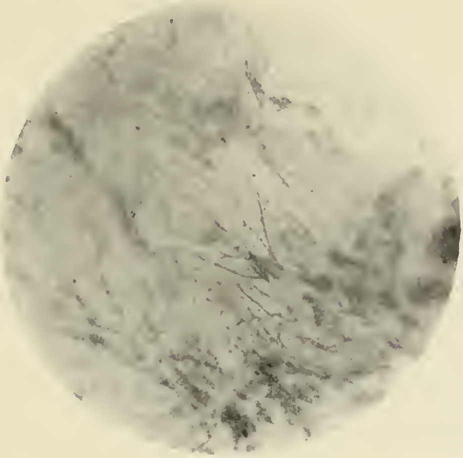


FIG. 6.

FIG. 7.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

264

Fig. 8.

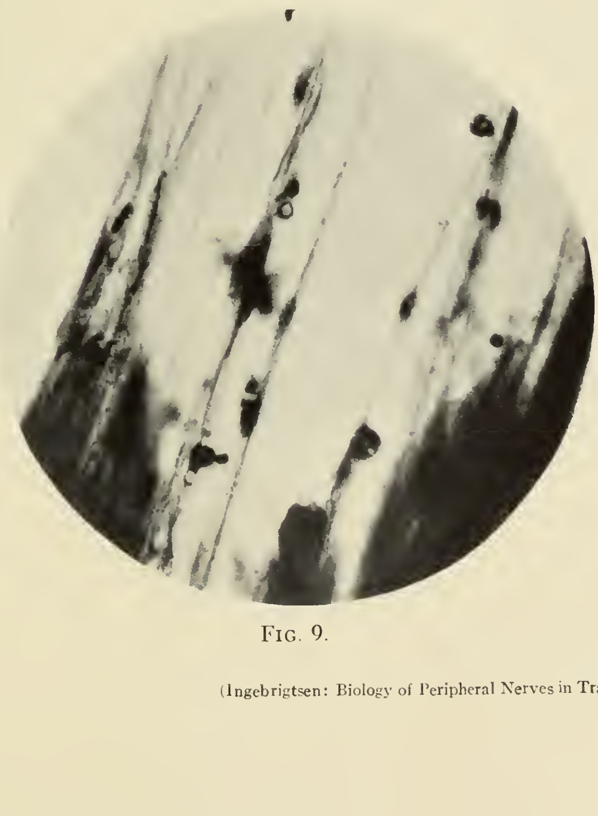


Fig. 9.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

264 6

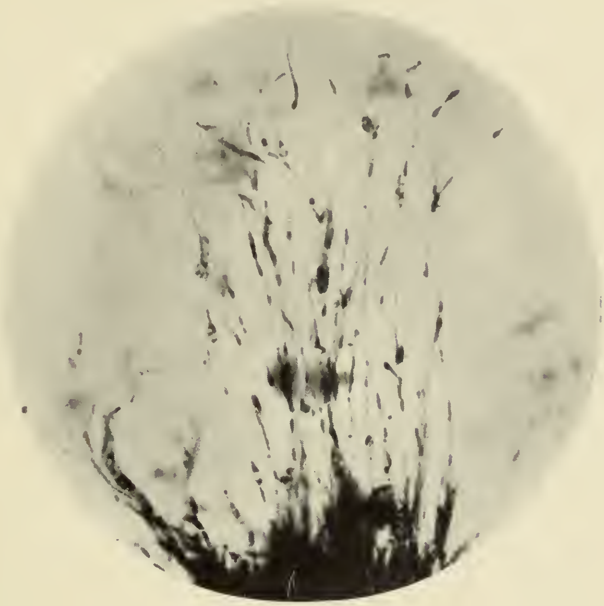


FIG 10.

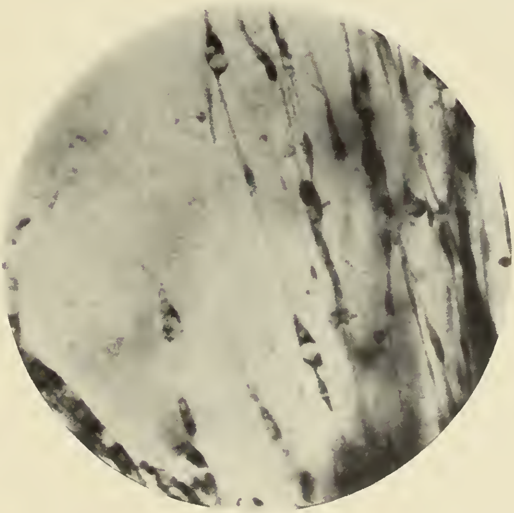


FIG. 11.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

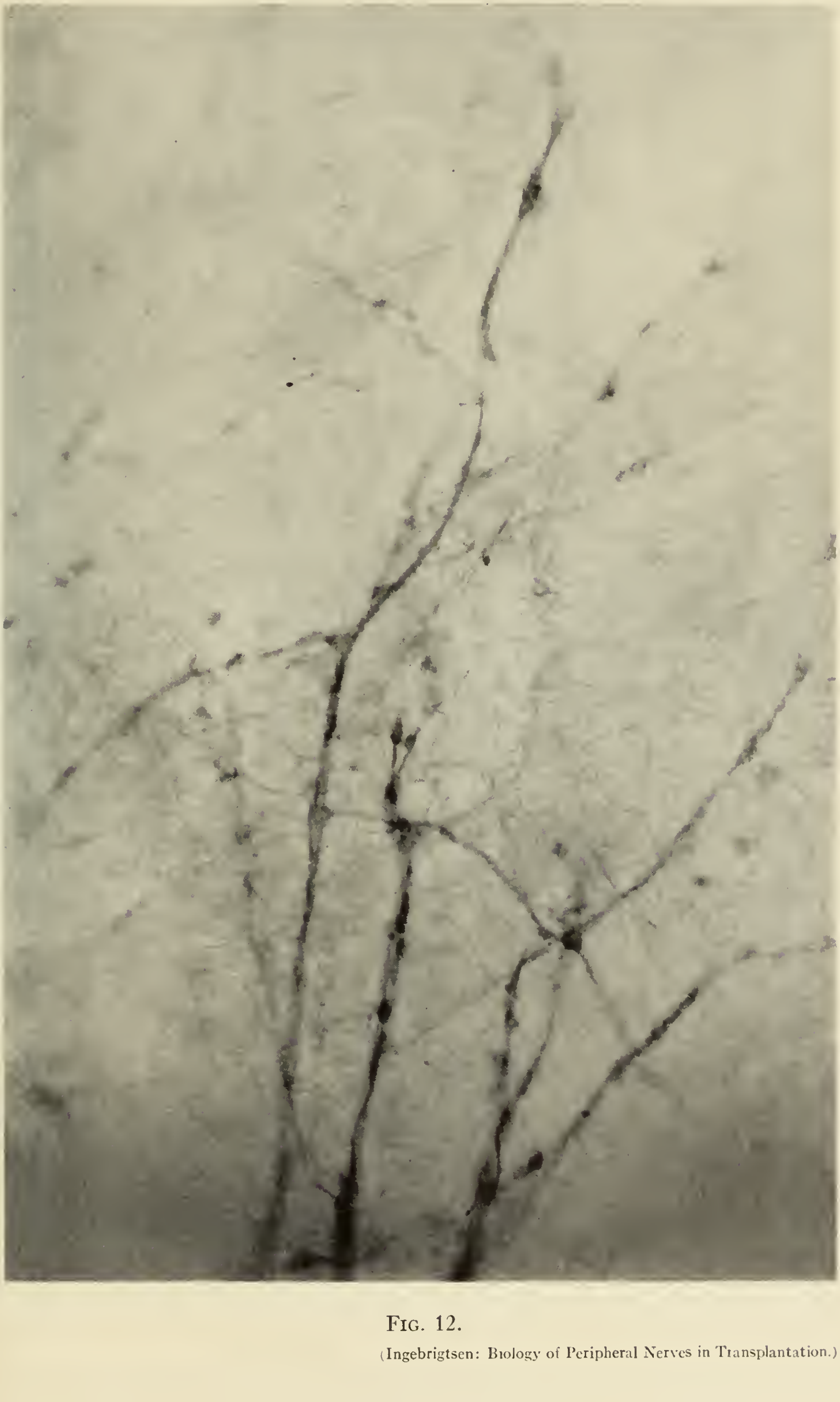


Fig. 12.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

264 8

FIG. 13.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

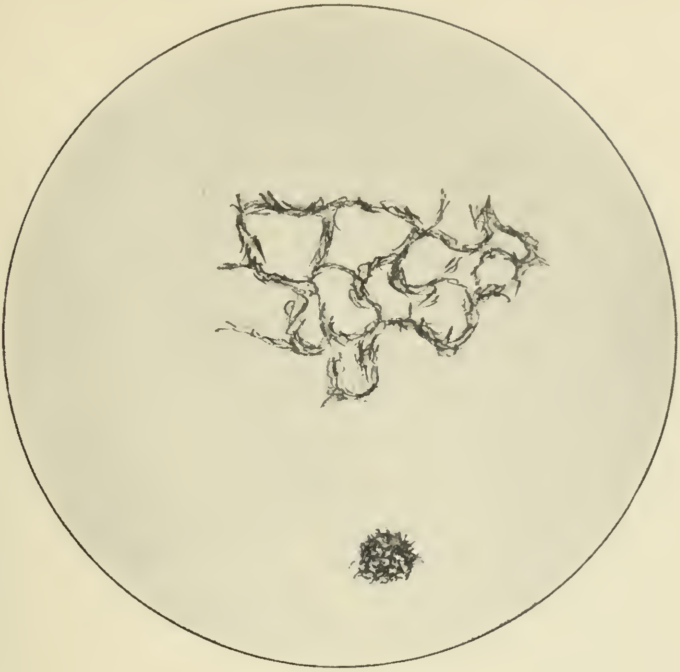


FIG. 1.

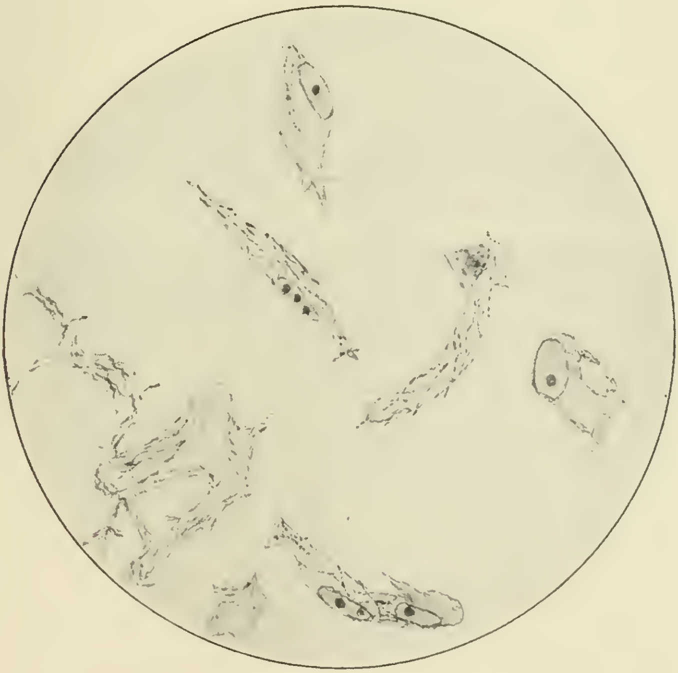


FIG. 2.

(Smyth: Bacteria and Animal Tissues. IV.)

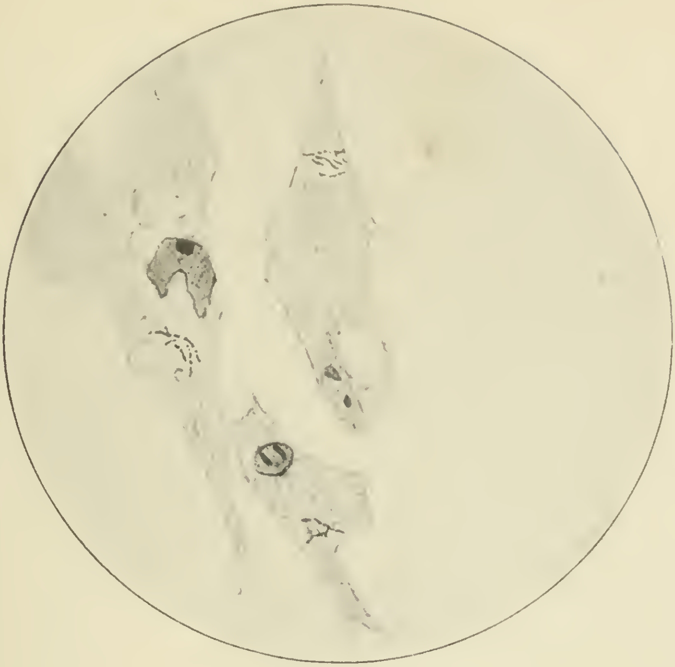


FIG. 3.

FIG. 4.

(Smyth: Bacteria and Animal Tissues. IV.)

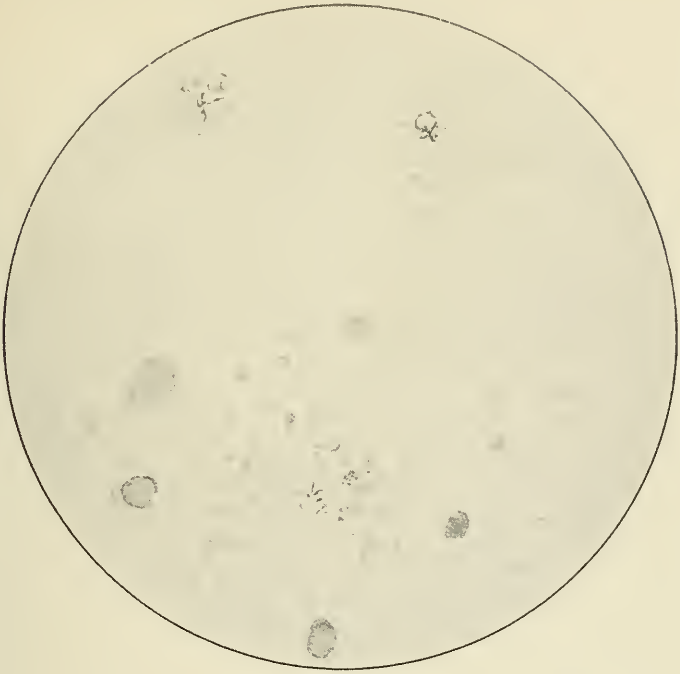


FIG. 5.

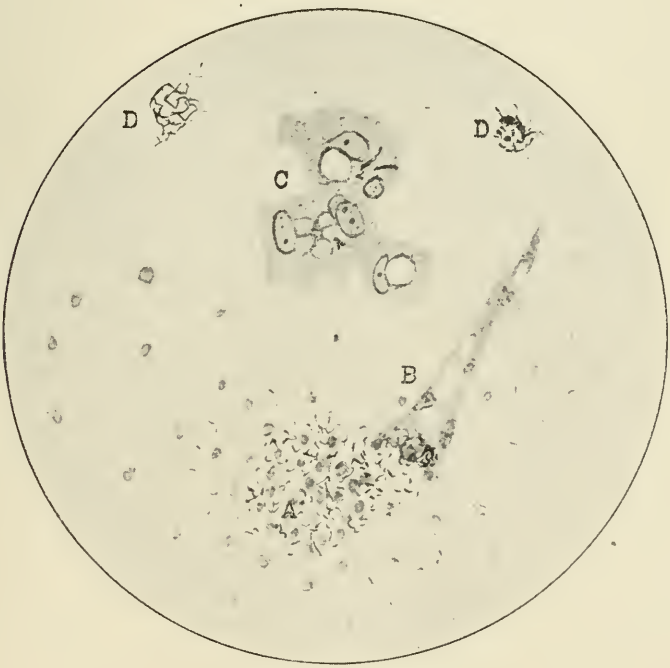


FIG. 6.

(Smyth: Bacteria and Animal Tissues. IV.)

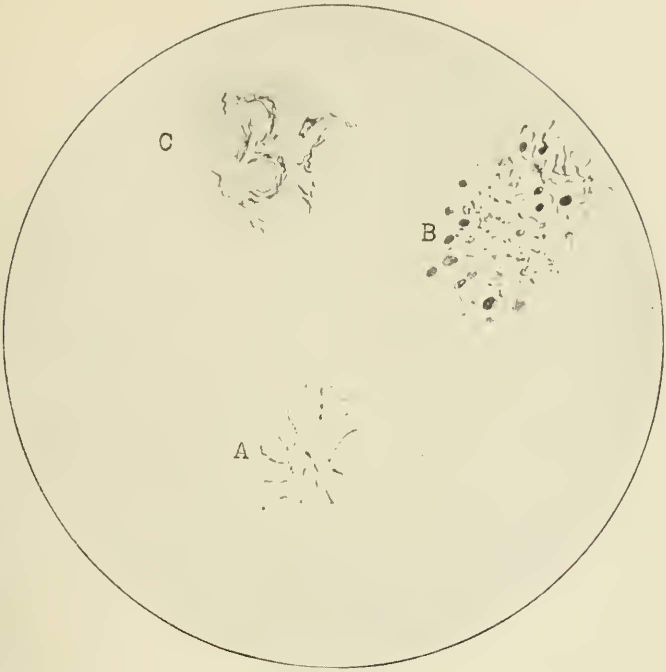


FIG. 7.

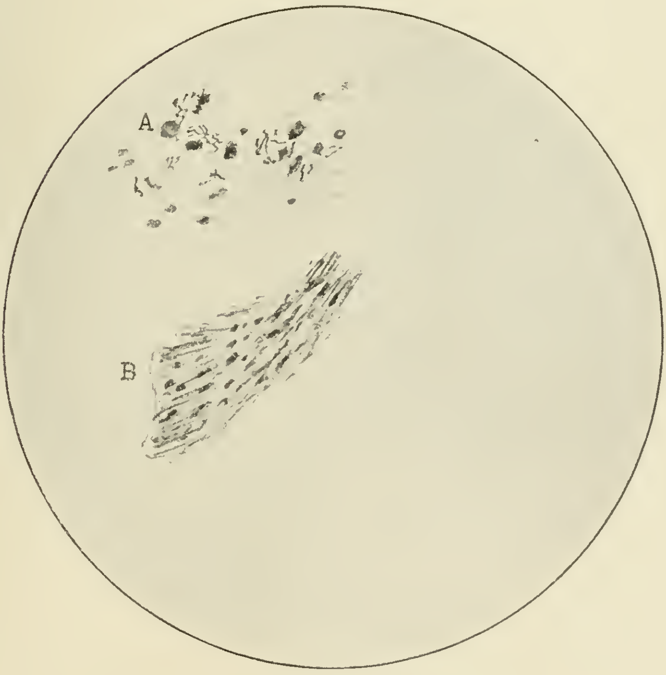


FIG. 8.

(Smyth: Bacteria and Animal Tissues. IV.)

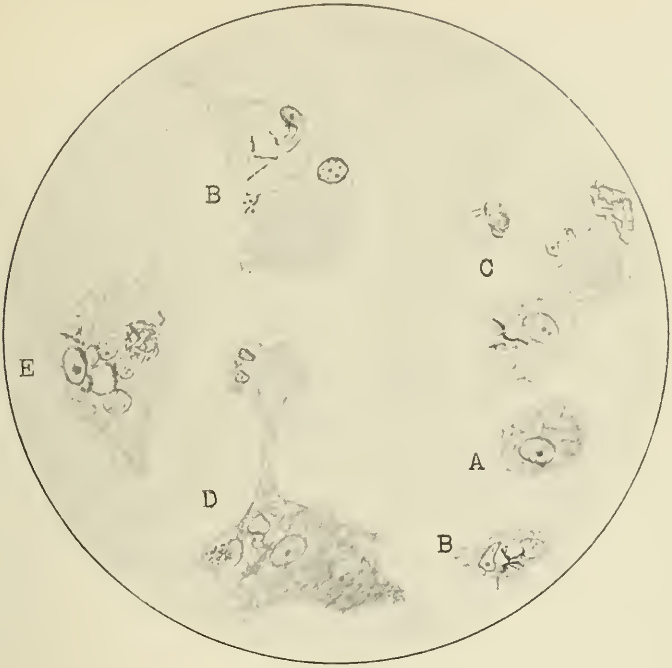


FIG. 9.

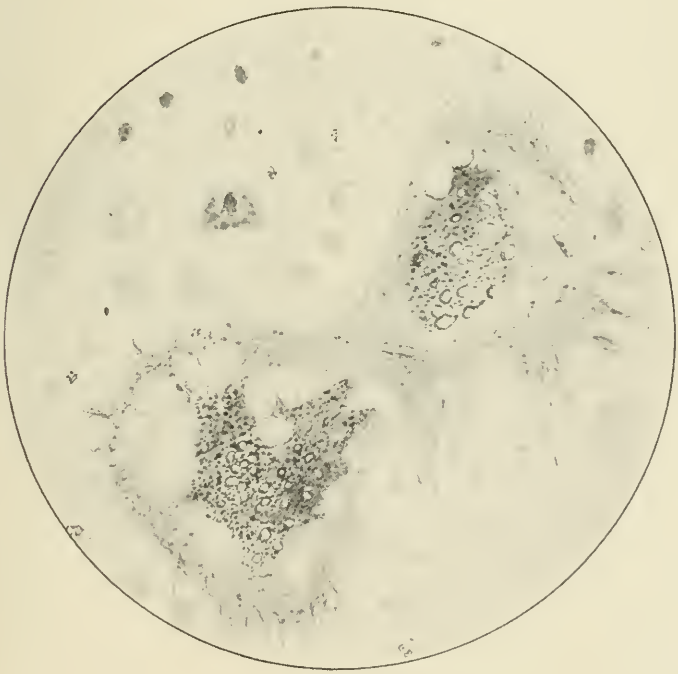


FIG. 10.

(Smyth: Bacteria and Animal Tissues. IV.)

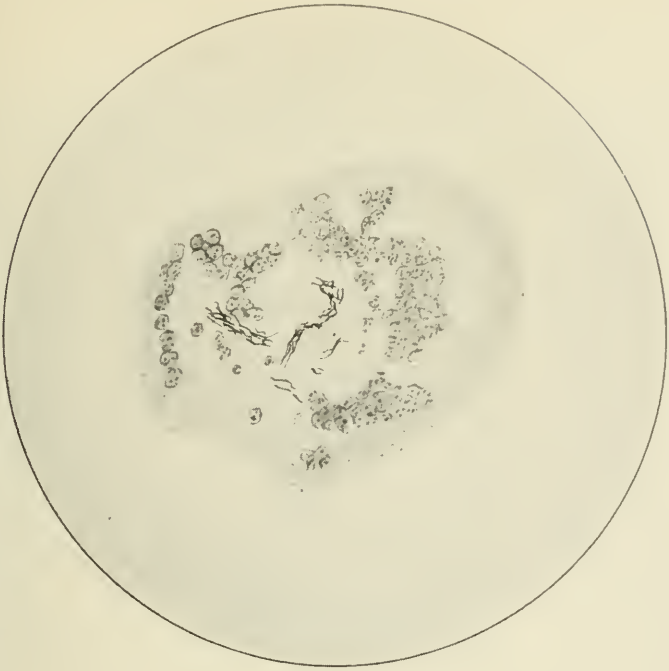


Fig. 11.

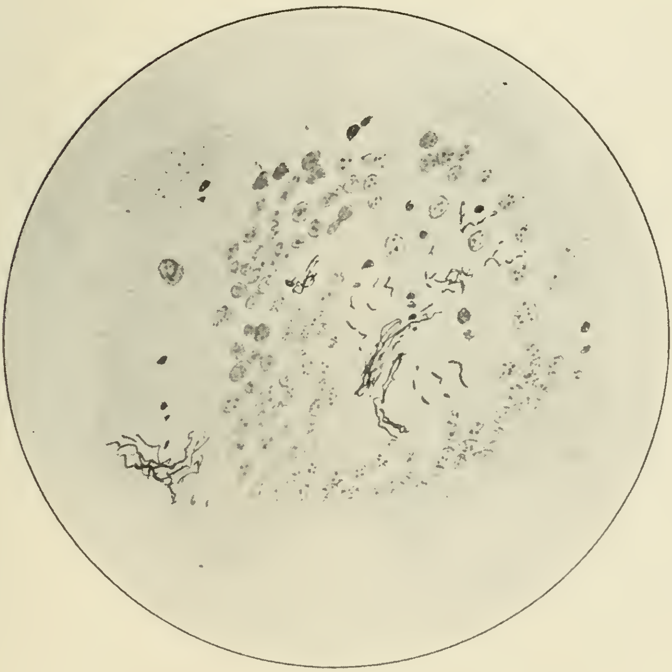
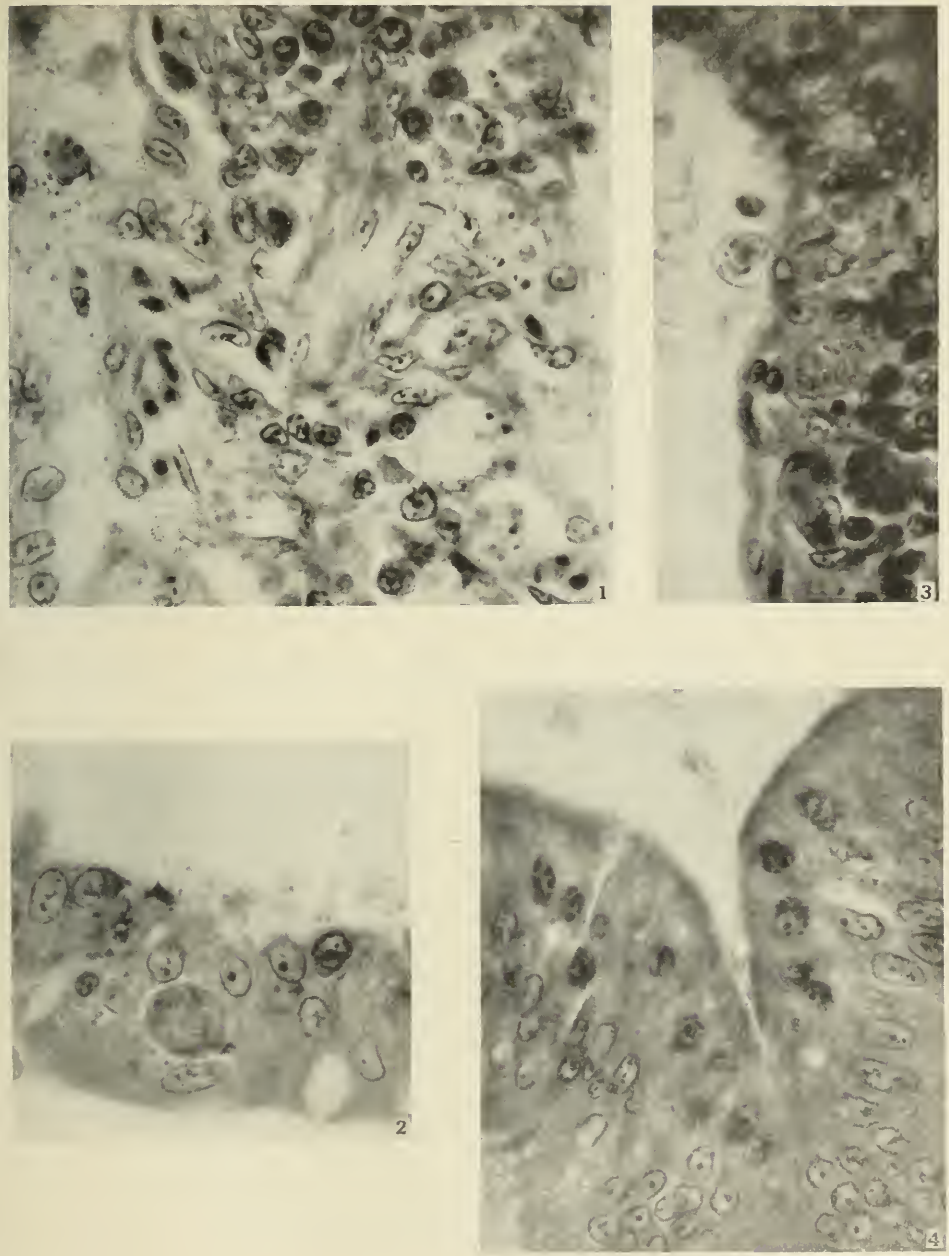


Fig. 12.

(Smyth: Bacteria and Animal Tissues. IV.)



(Smith: Aberrant Intestinal Protozoan Parasites.)

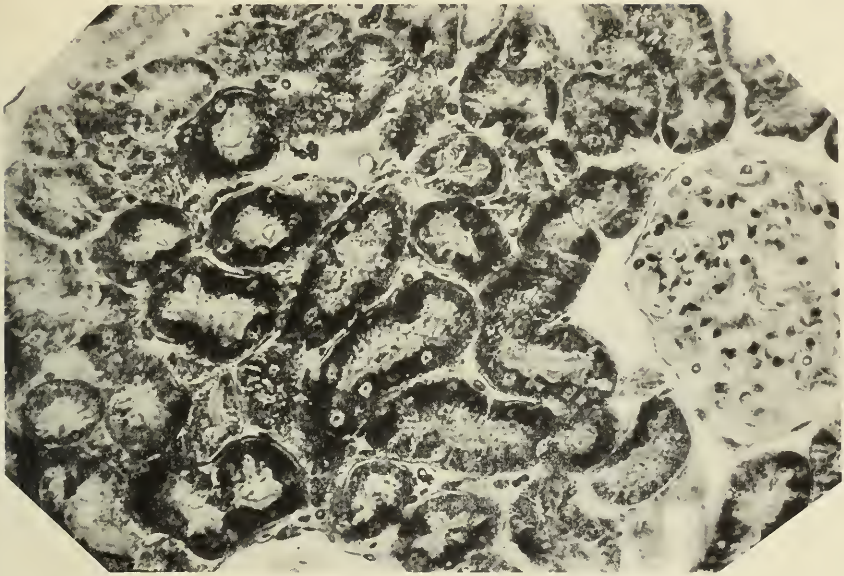


Fig. 1.

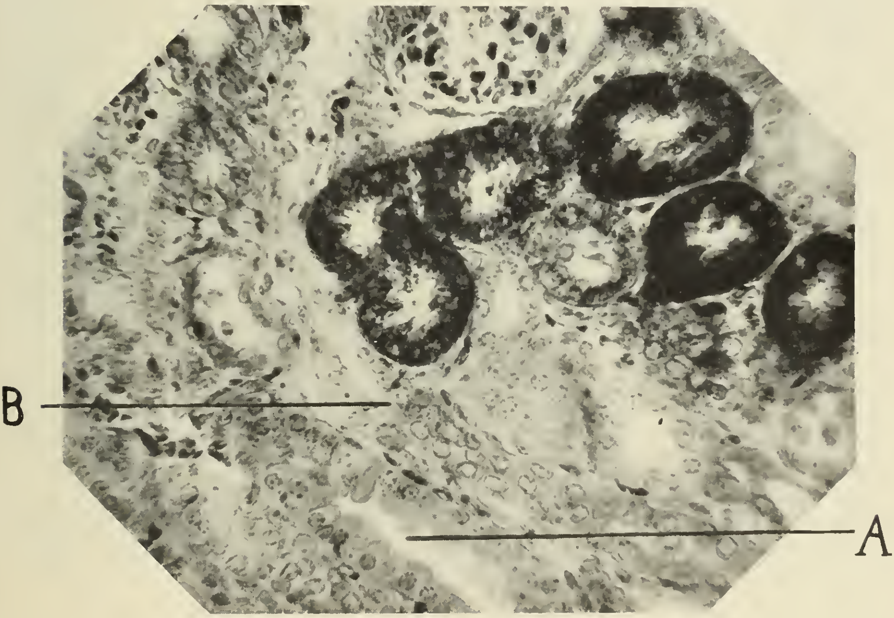


Fig. 2.

(Oliver: Chronic Uranium Nephritis.)

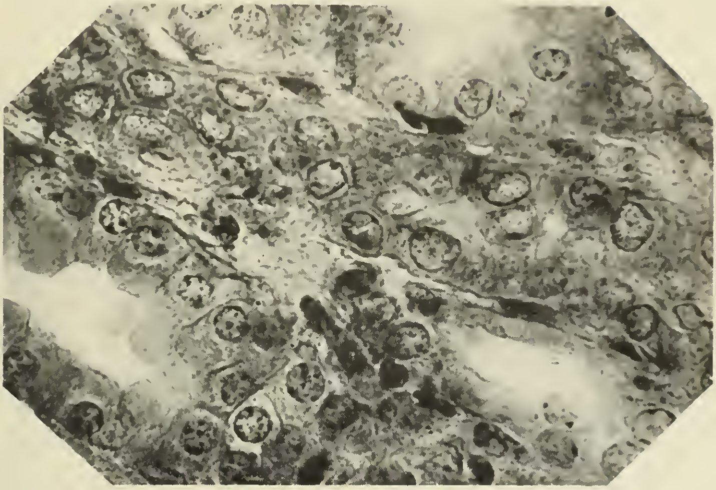


FIG. 3.

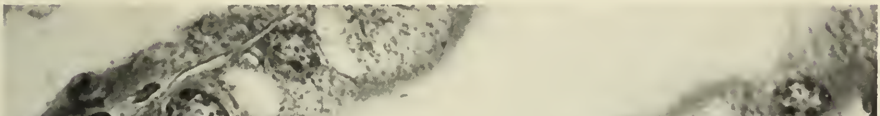


FIG. 4.

(Oliver: Chronic Uranium Nephritis.)

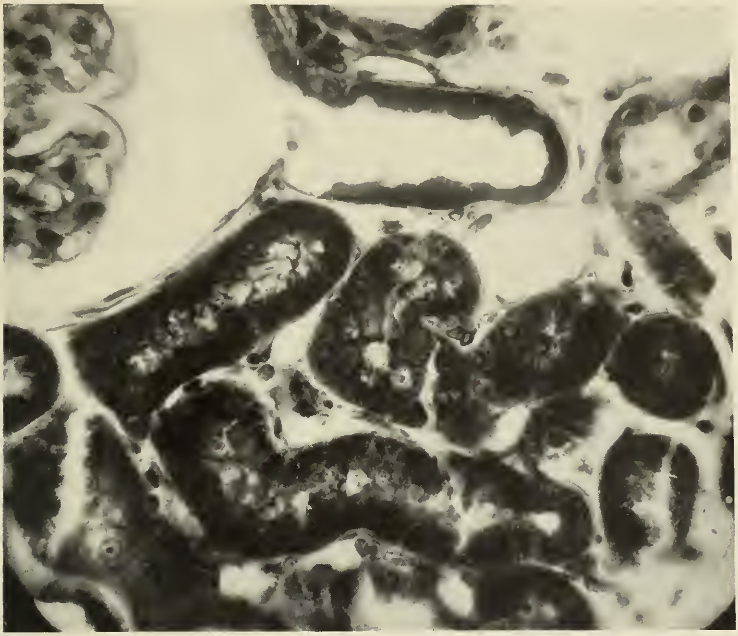


FIG. 5.

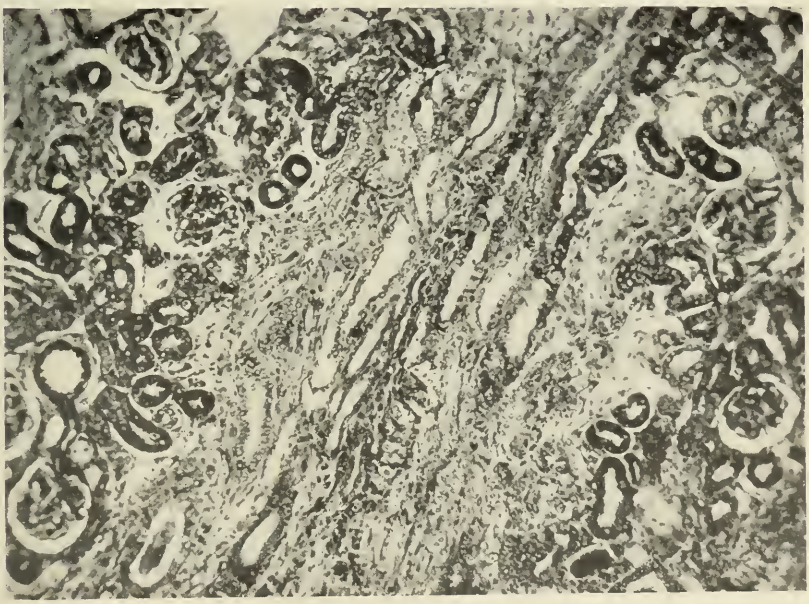


FIG. 6.

(Oliver: Chronic Uranium Nephritis.)

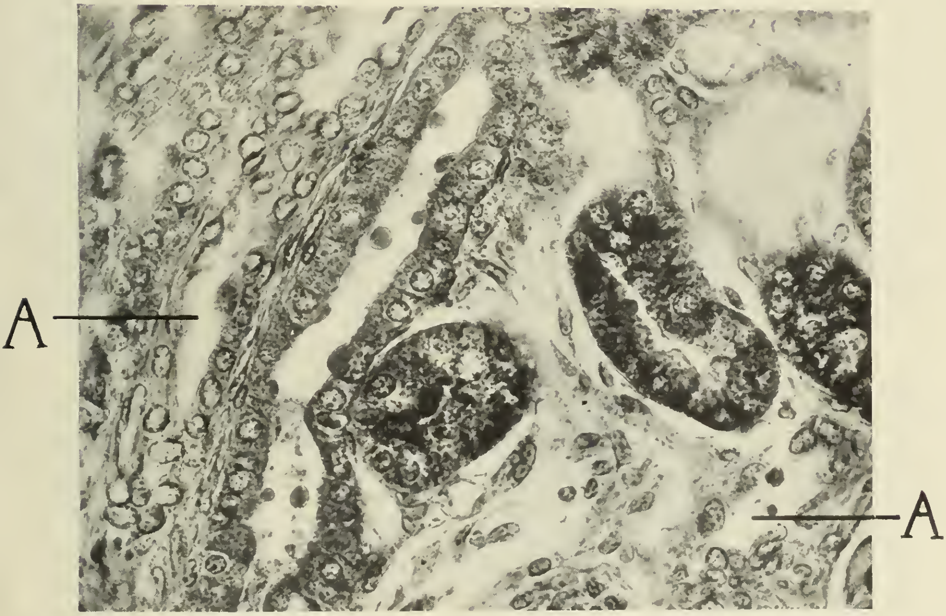
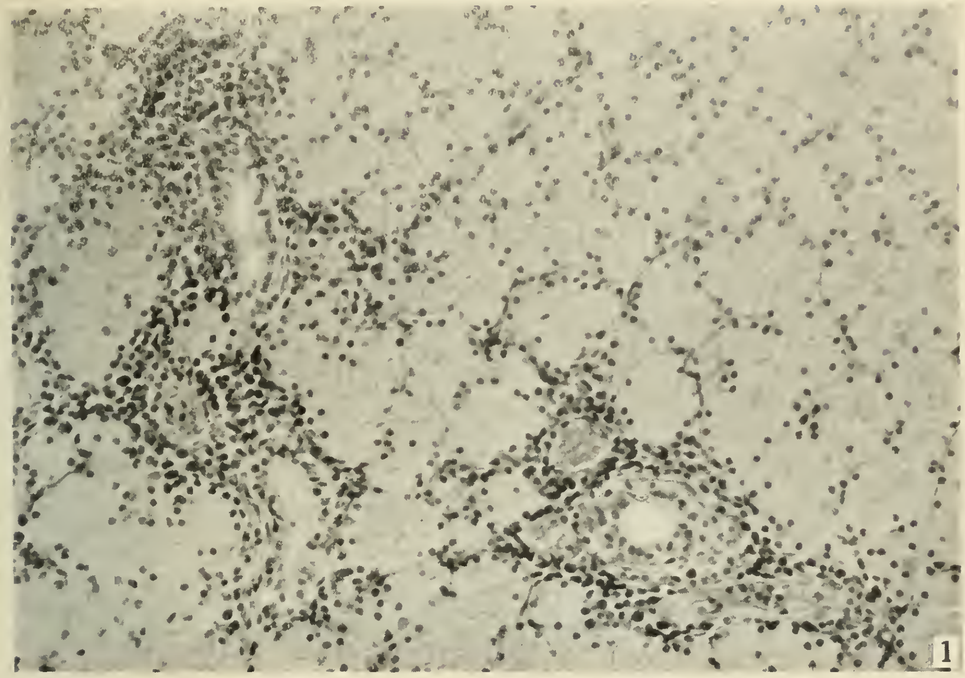
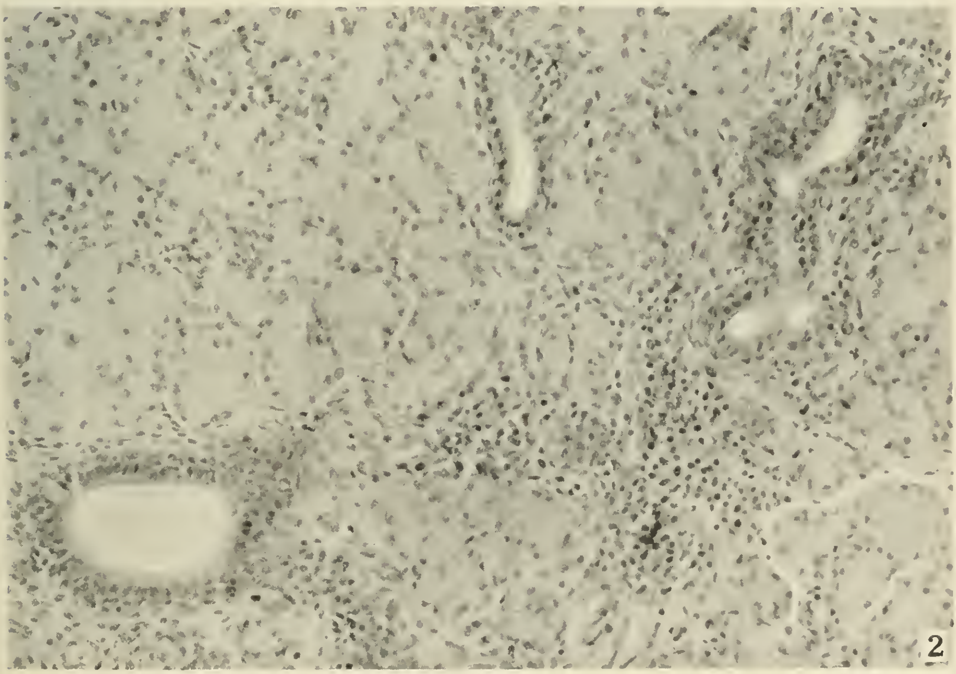


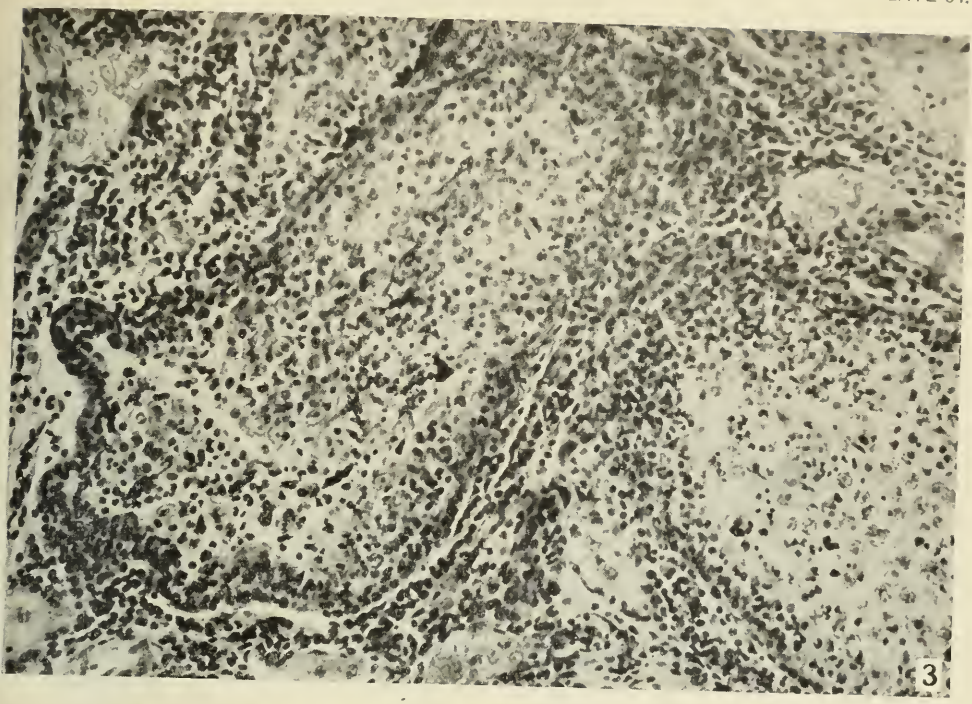
FIG. 7.

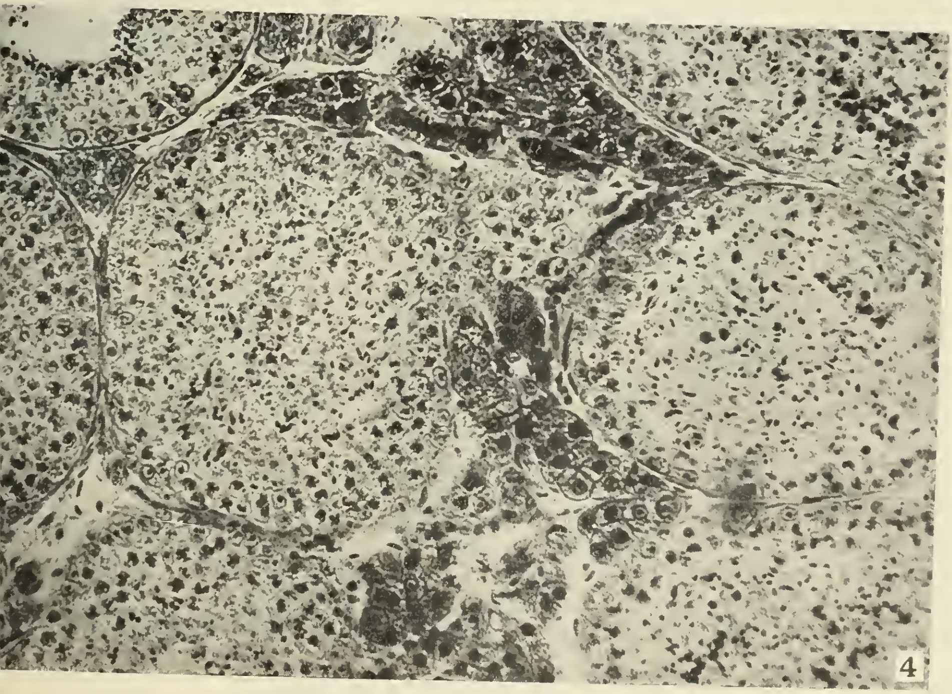
376'





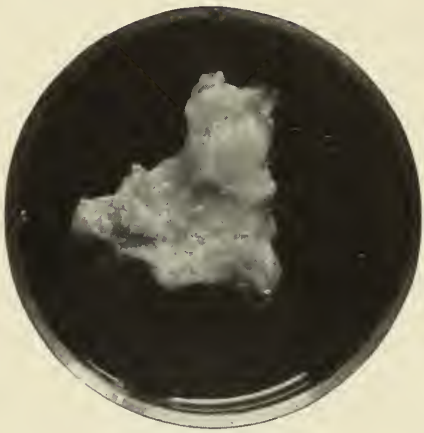
(Wollstein: Experimental Study of Parotitis (Mumps).)





(Wollstein: Experimental Study of Parotitis (Mumps).)

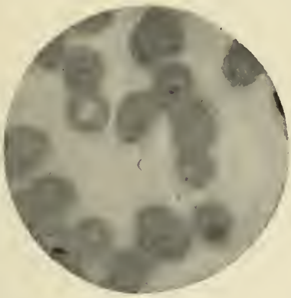
Left parotid, inoculated.

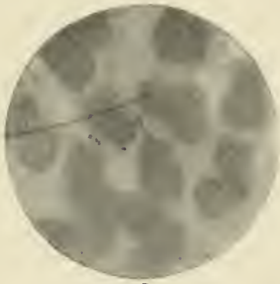


Right parotid, not inoculated.

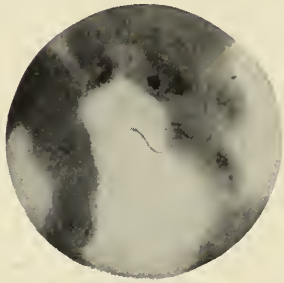
Fig. 5.

402'


1


2

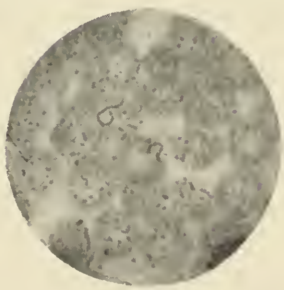

3


4

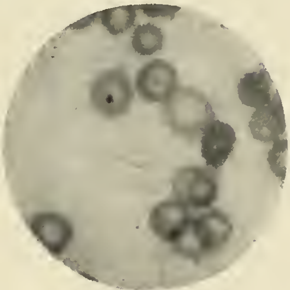

5

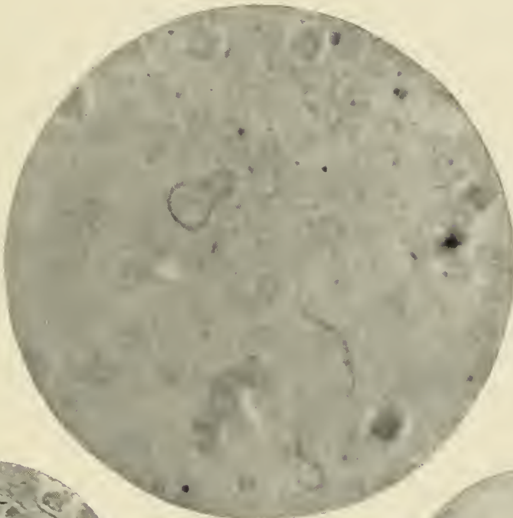

6


7

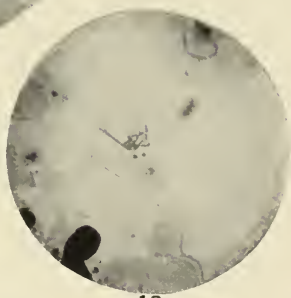

8

(Inada, Ido, Hoki, Kaneko and Ito: Weil's Disease.)


9


10


11


12


13


14

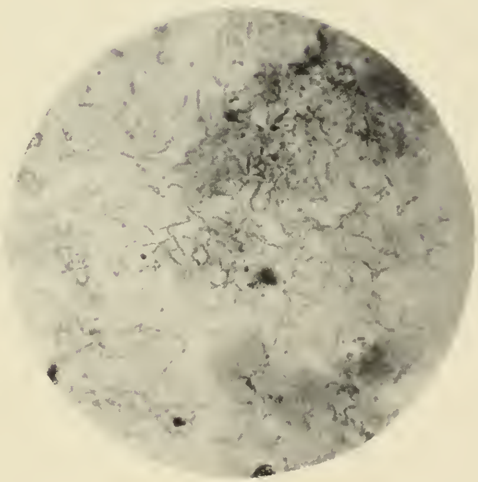
(Inada, Ido, Hoki, Kaneko and Ito: Weil's Disease.)

4 02

15

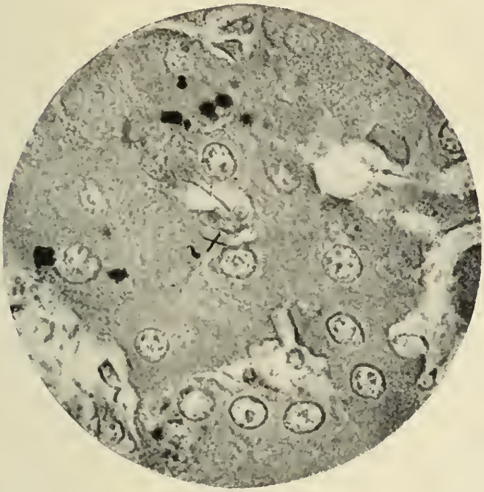
16

17



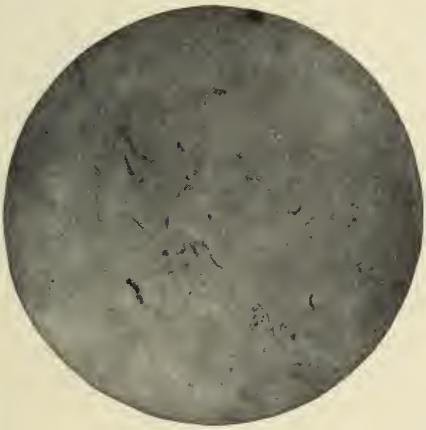
18

(Inada, Ido, Hoki, Kaneko and Ito: Weil's Disease.)

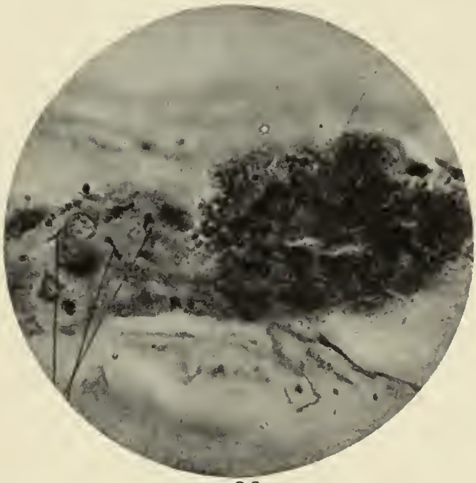


19

20



21



22

(Inada, Ido, Hoki, Kaneko and Ito: Weil's Disease.)

402

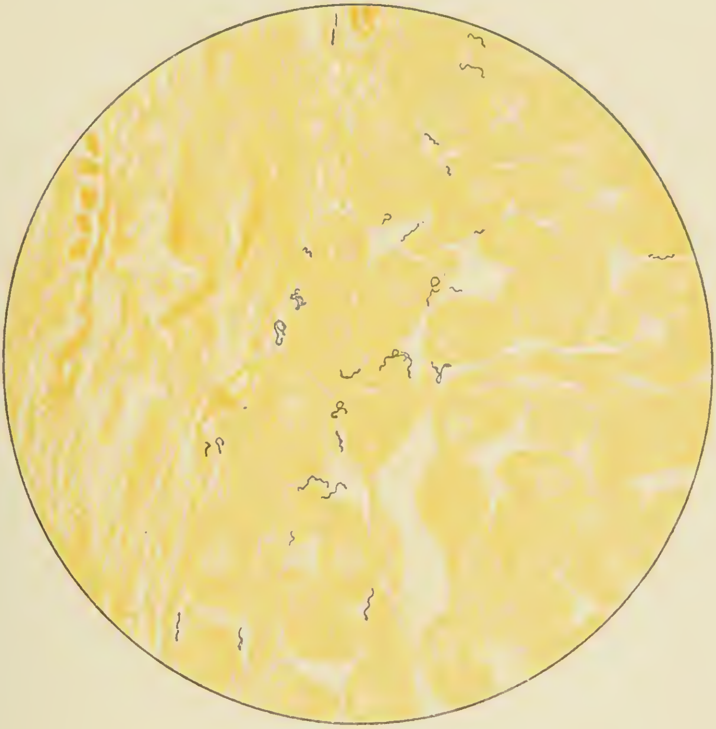
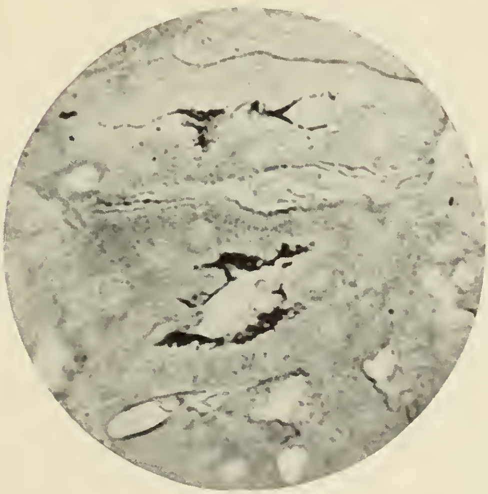


FIG. 23.



24

(Inada, Ido, Hoki, Kaneko and Ito: Weil's Disease.)

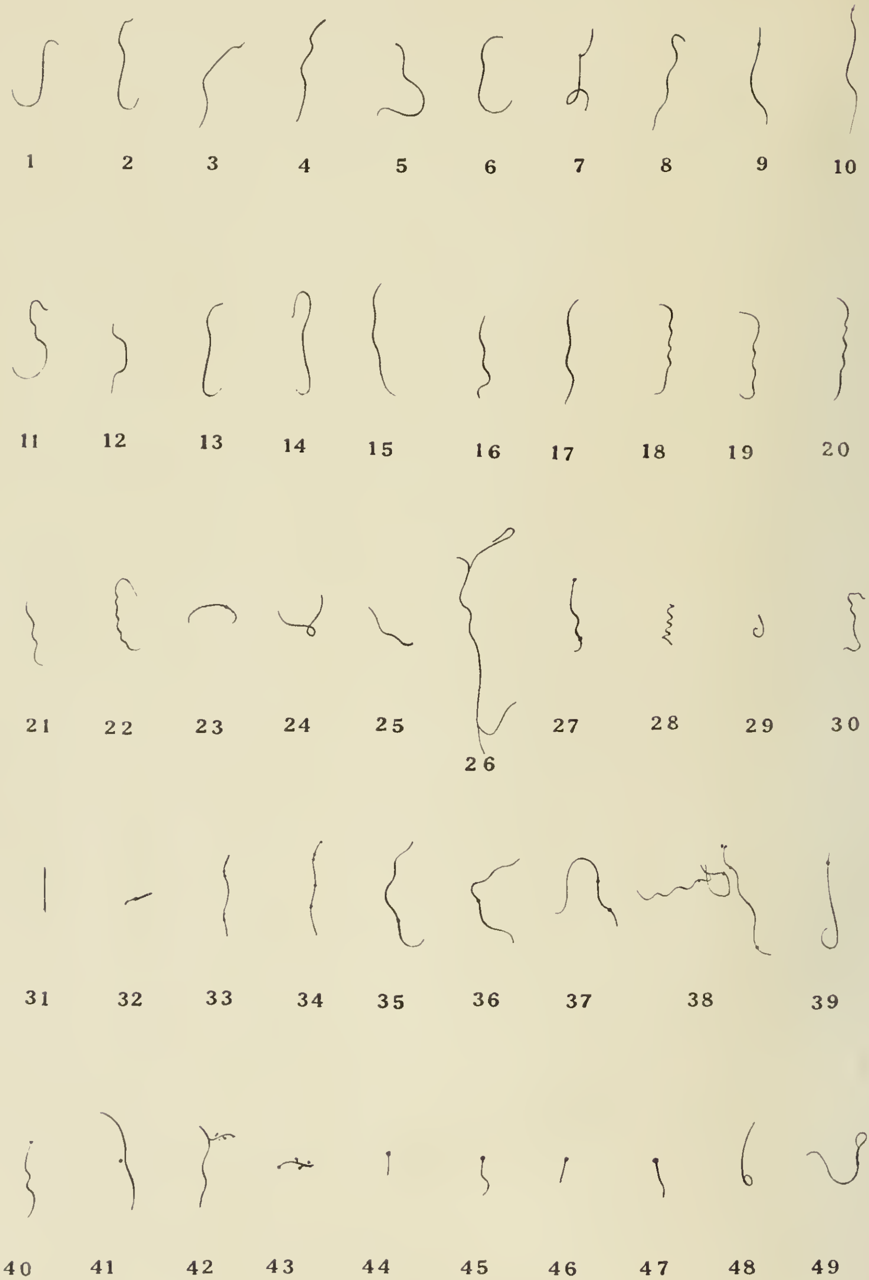

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49

Fig. 25 a.

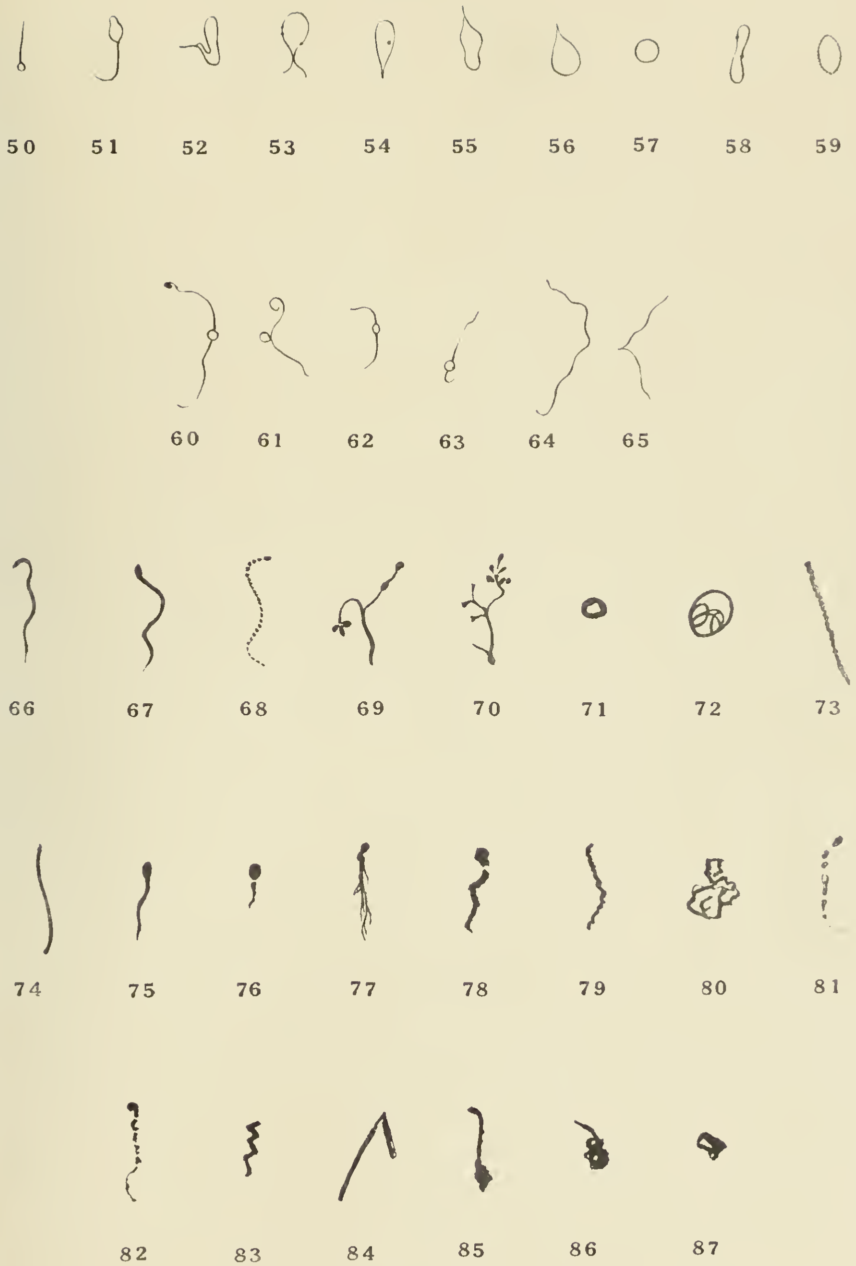


Fig. 25 b.

(Inada, Ido, Hoki, Kaneko and Ito: Weil's Disease.)

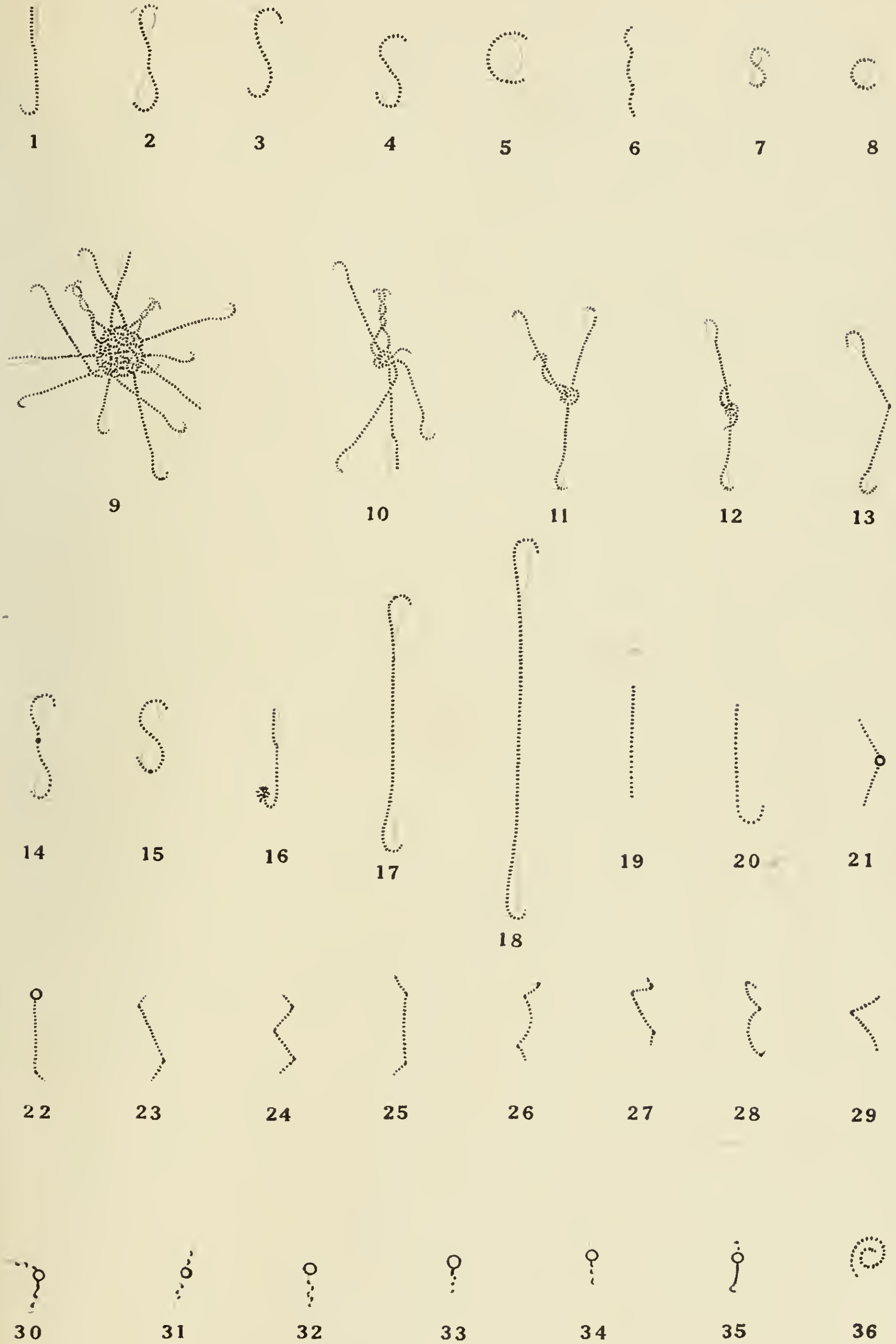
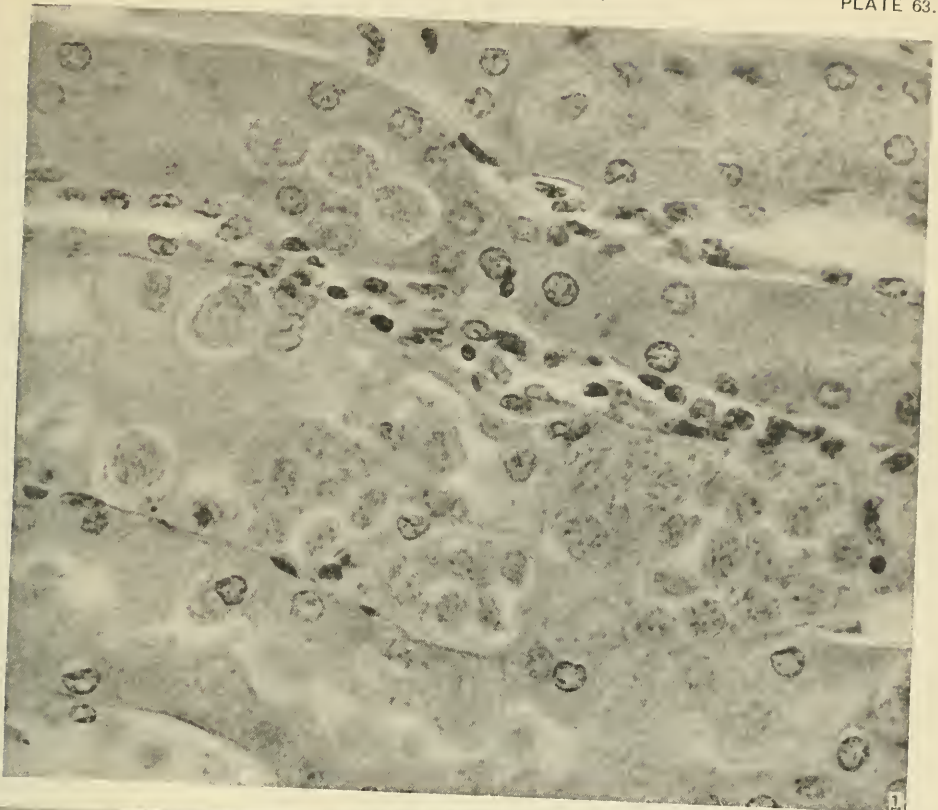


FIG. 26.

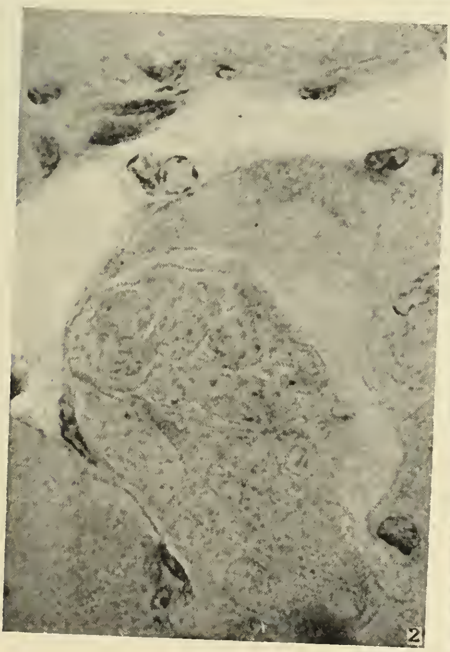
(Inada, Ido, Hoki, Kaneko and Ito: Weil's Disease.)

442'

PLATE 63.



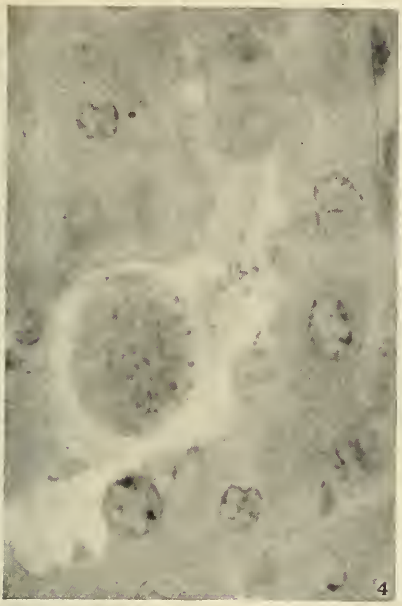
1

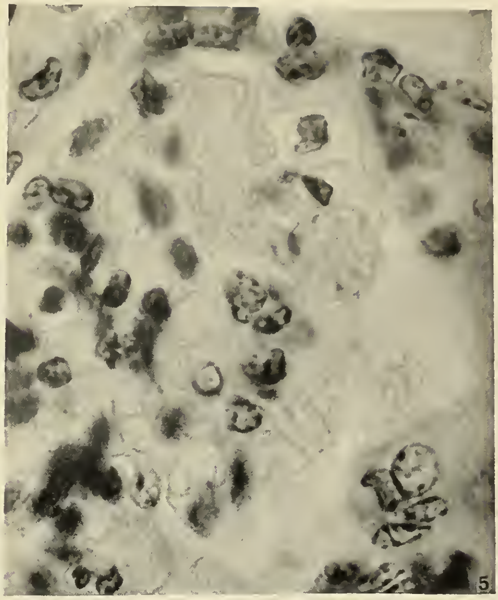


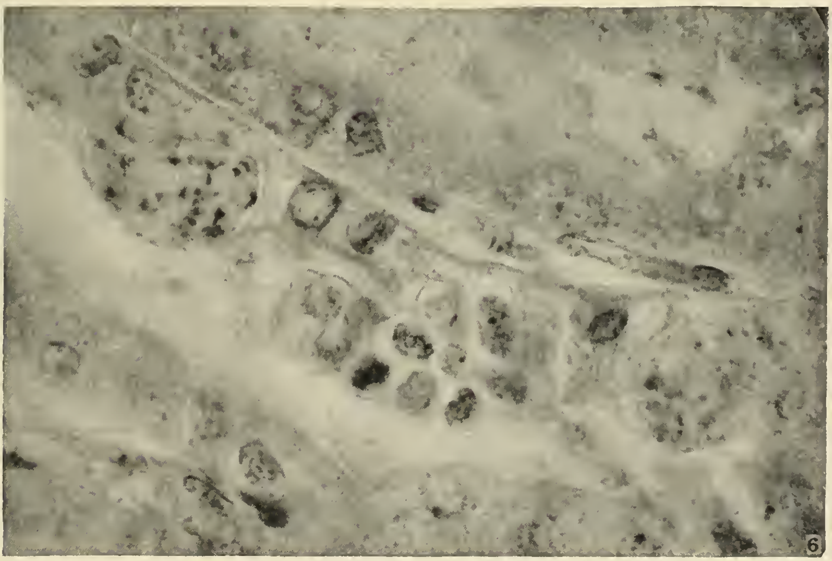
2

3

(Pearce: Klosiella Infection of the Guinea Pig.)

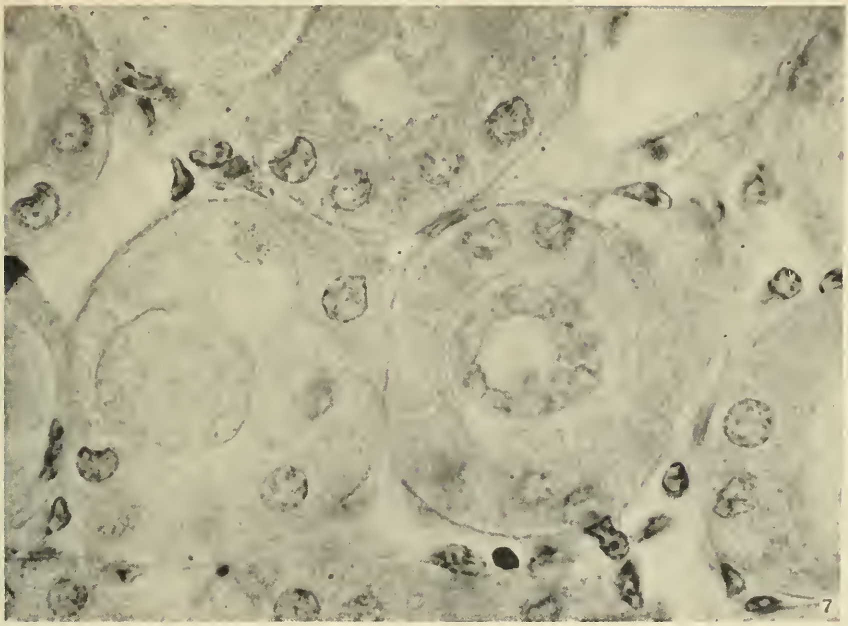






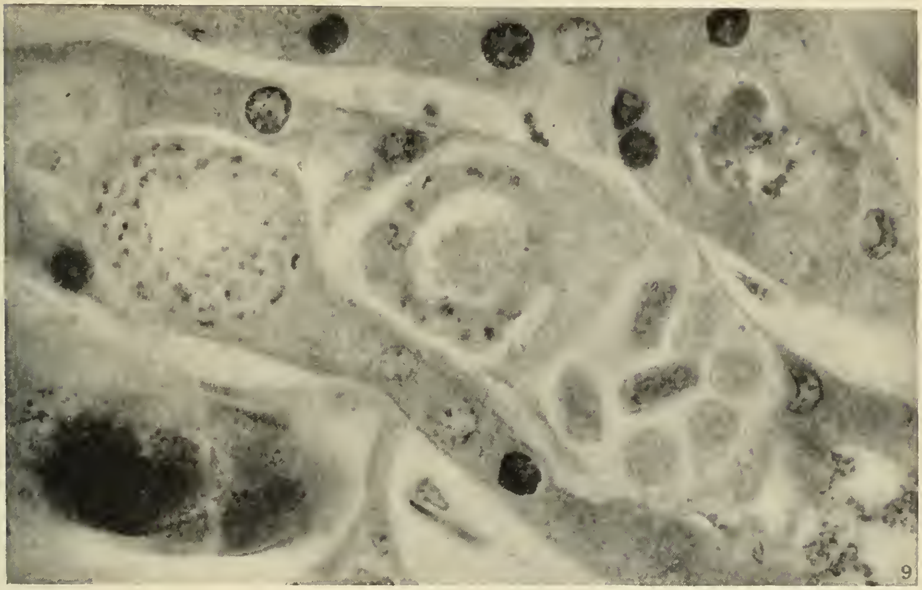
(Pearce: Klosiella Infection of the Guinea Pig.)

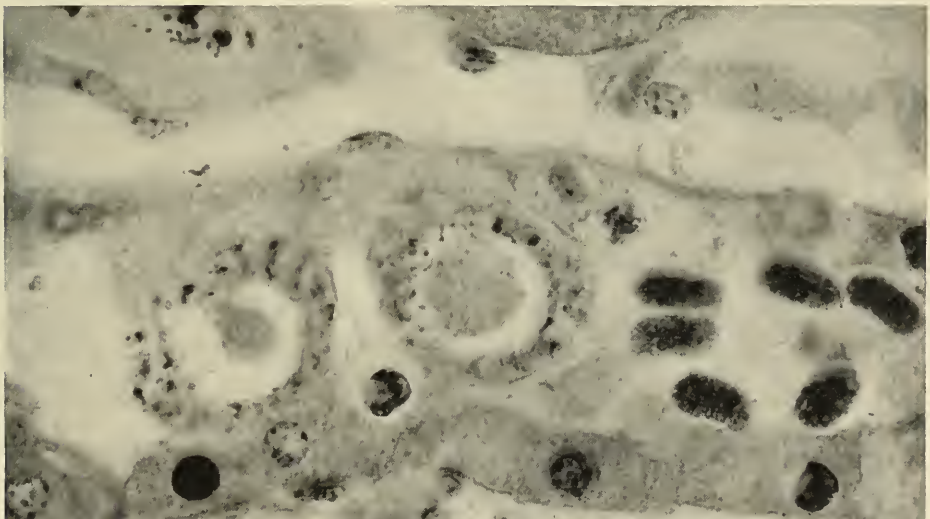
442



(Pearce: Klosiella Infection of the Guinea Pig.)

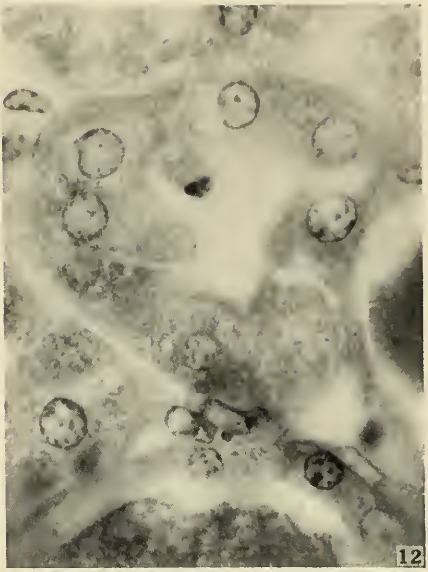
442[4]

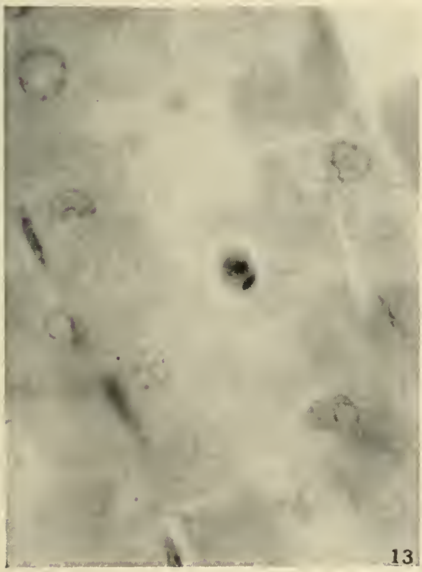


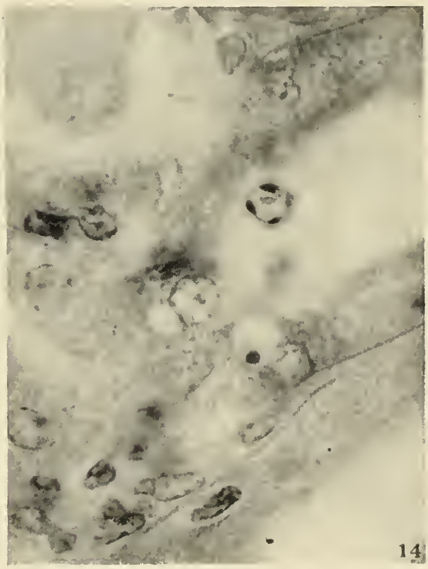


(Pearce: Klosiella Infection of the Guinea Pig.)

442

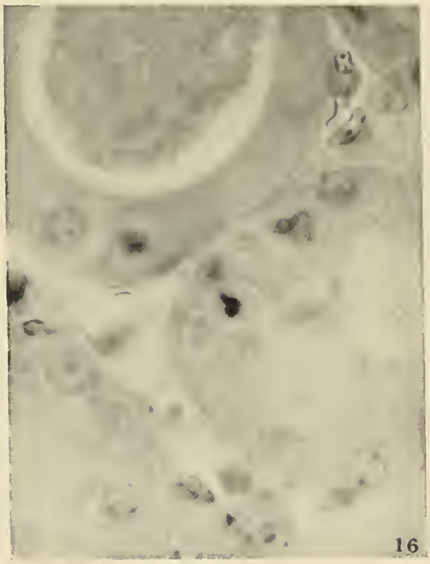




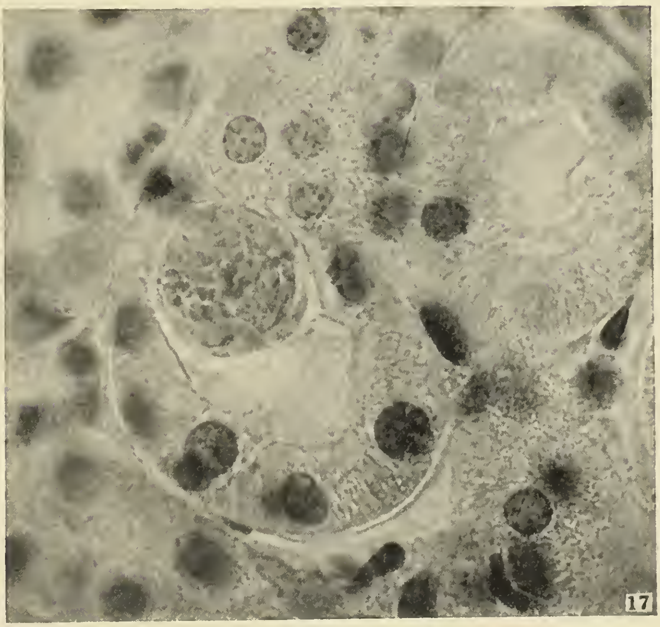


(Pearce: Klosiella Infection of the Guinea Pig.)

15

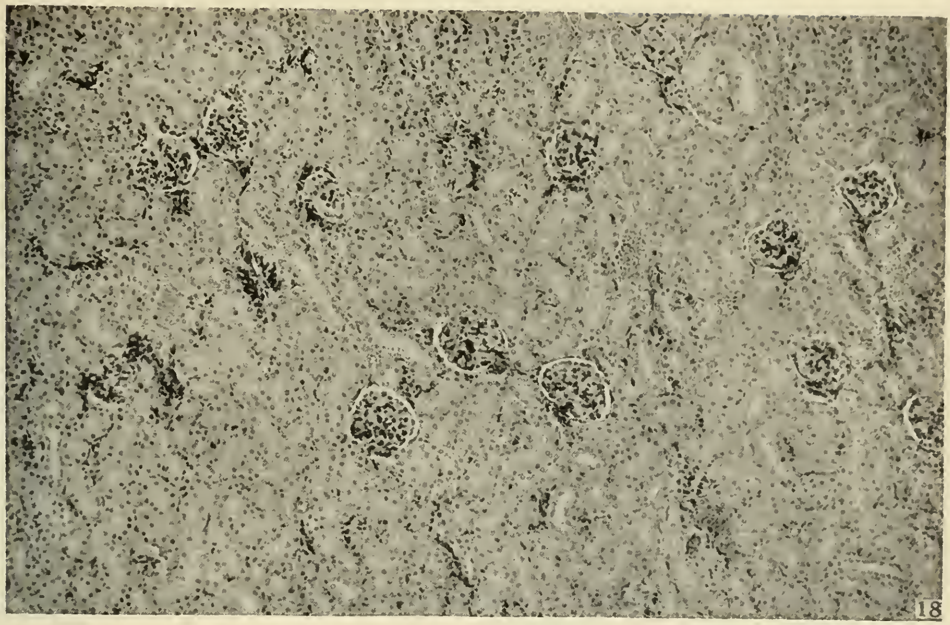


16

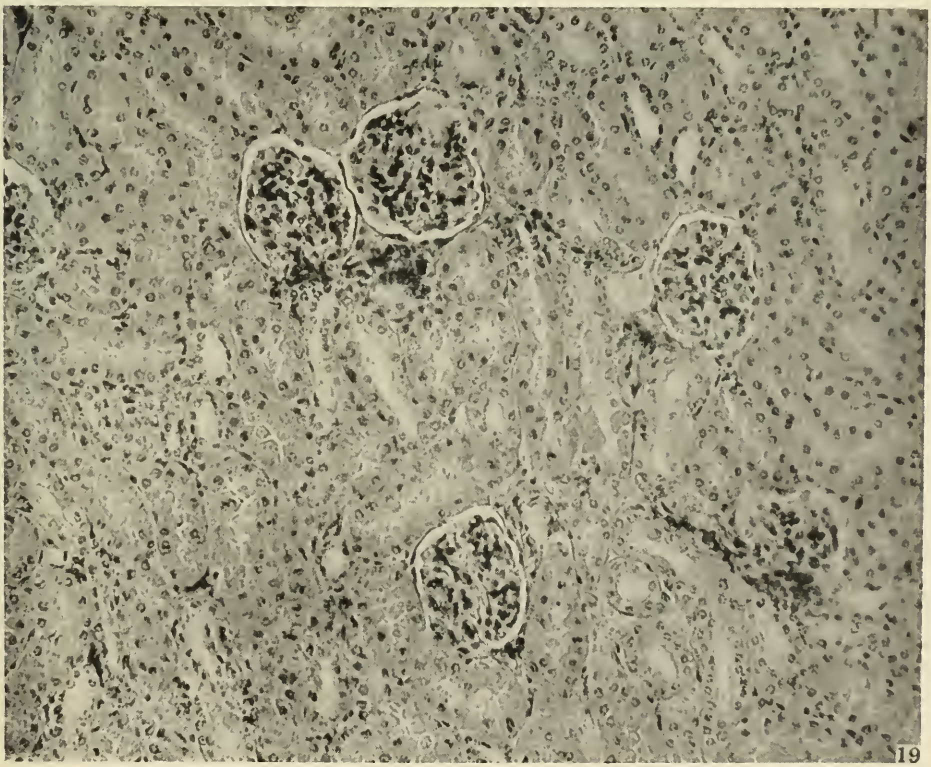


17

(Pearce: Klosiella Infection of the Guinea Pig.)



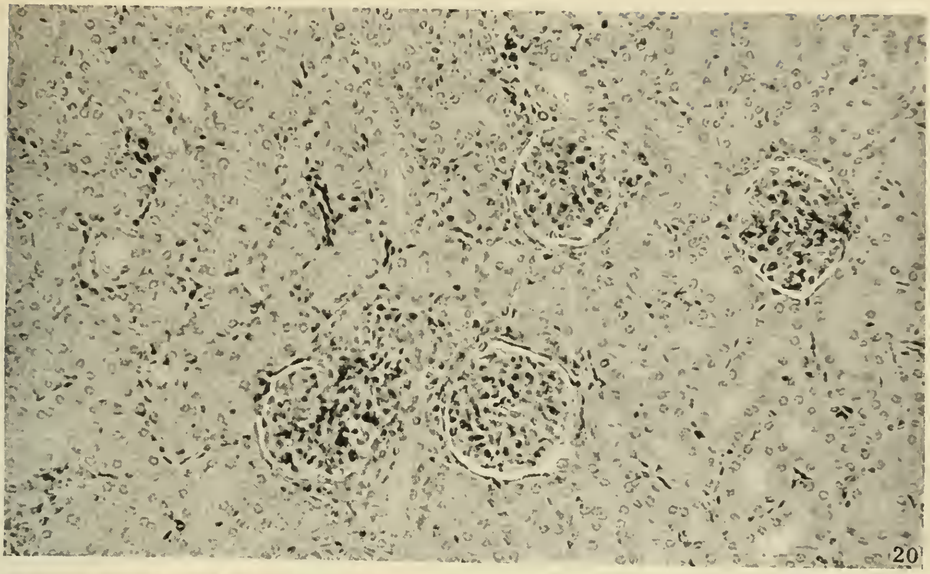
18

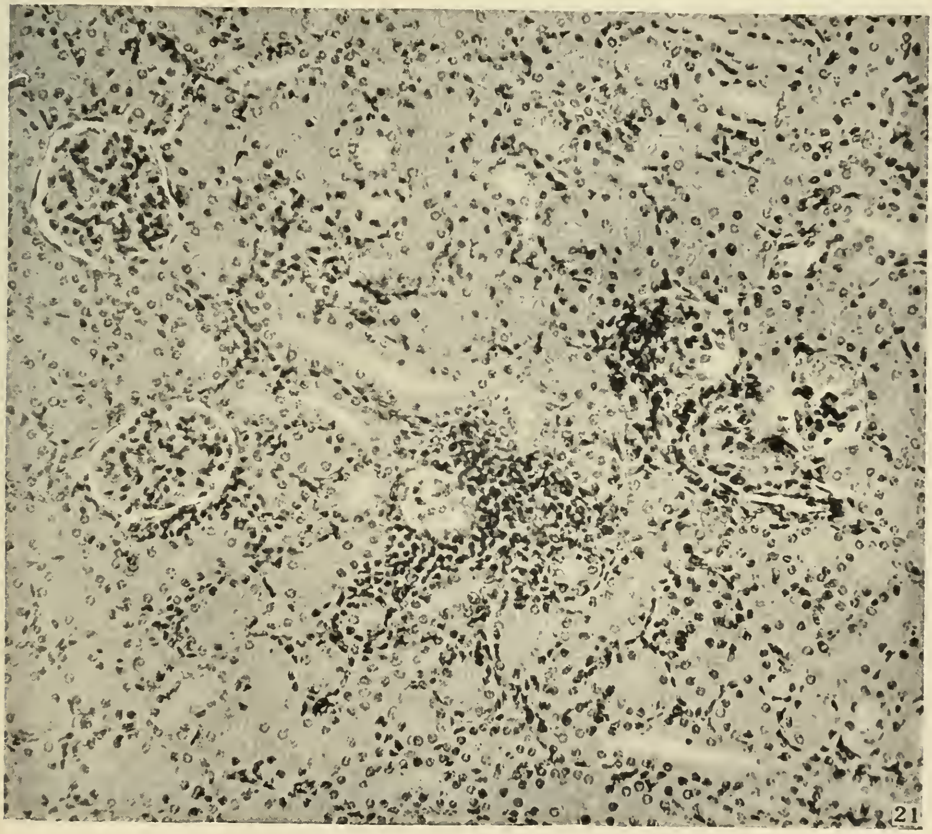


19

(Pearce: Klosiella Infection of the Guinea Pig.)

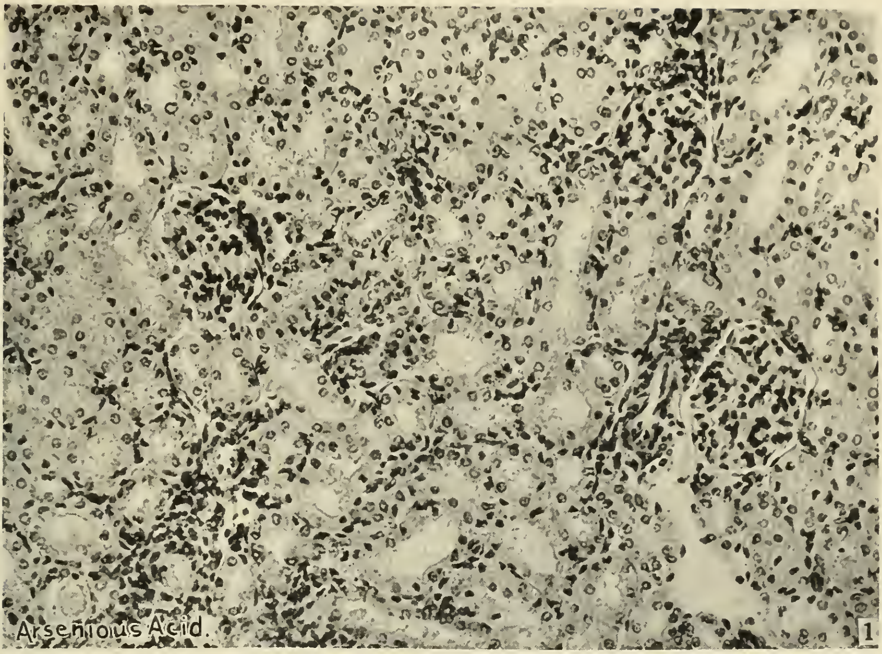
4428

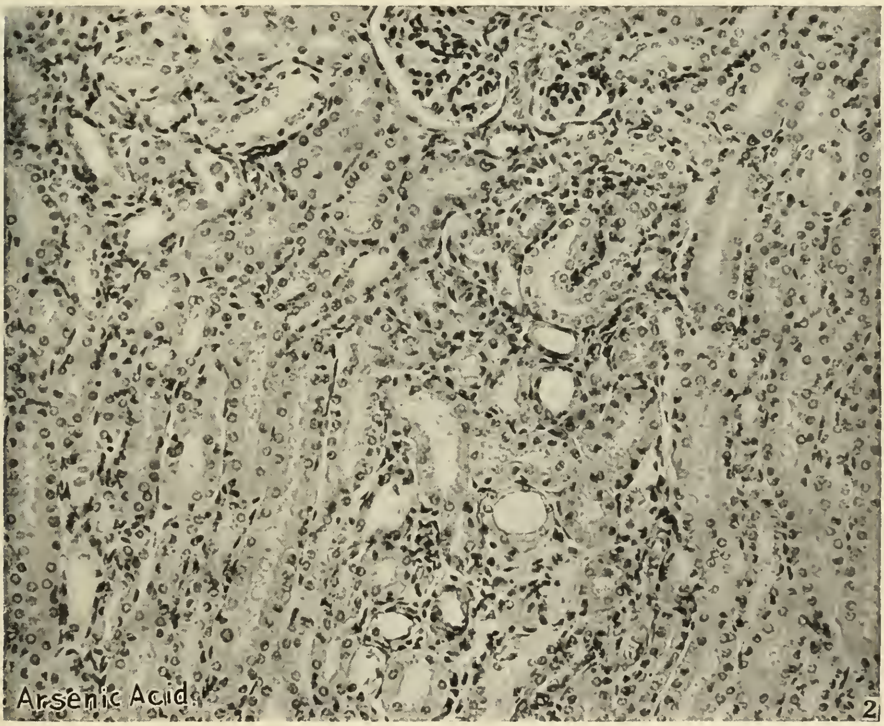




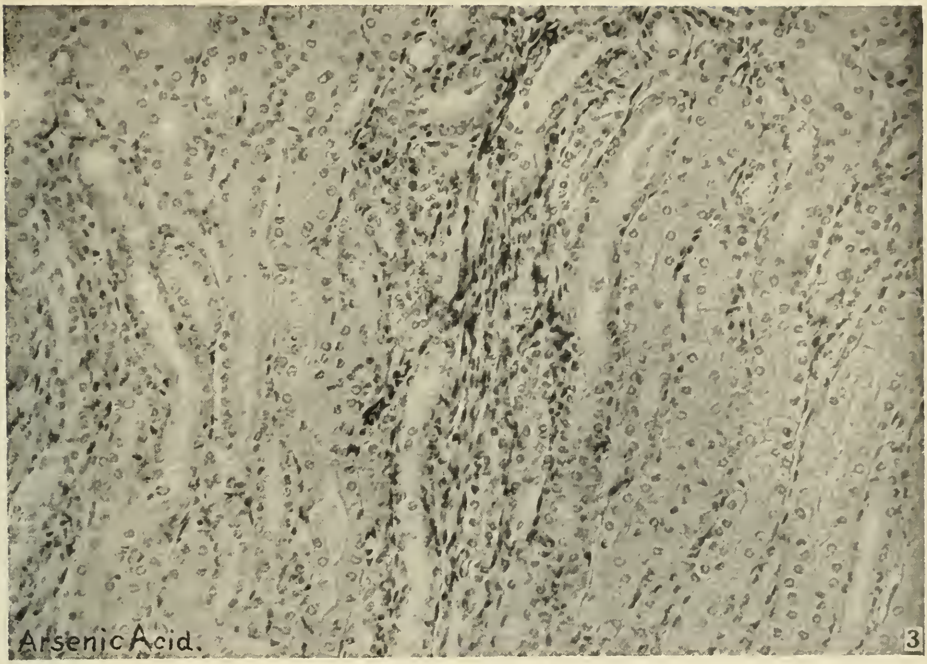
(Pearce: Klosiella Infection of the Guinea Pig.)

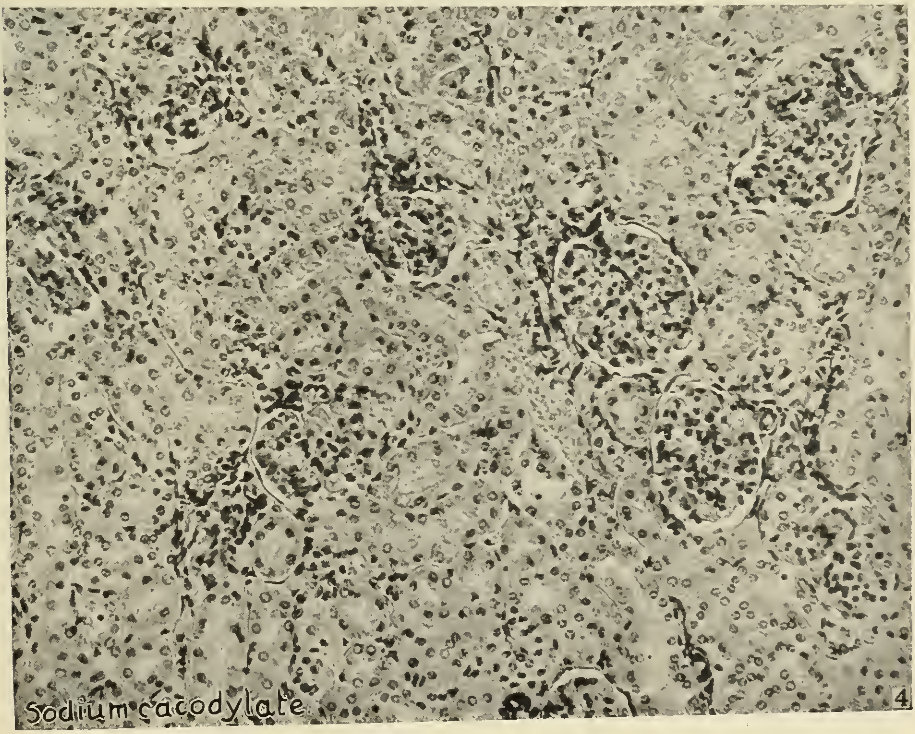
458





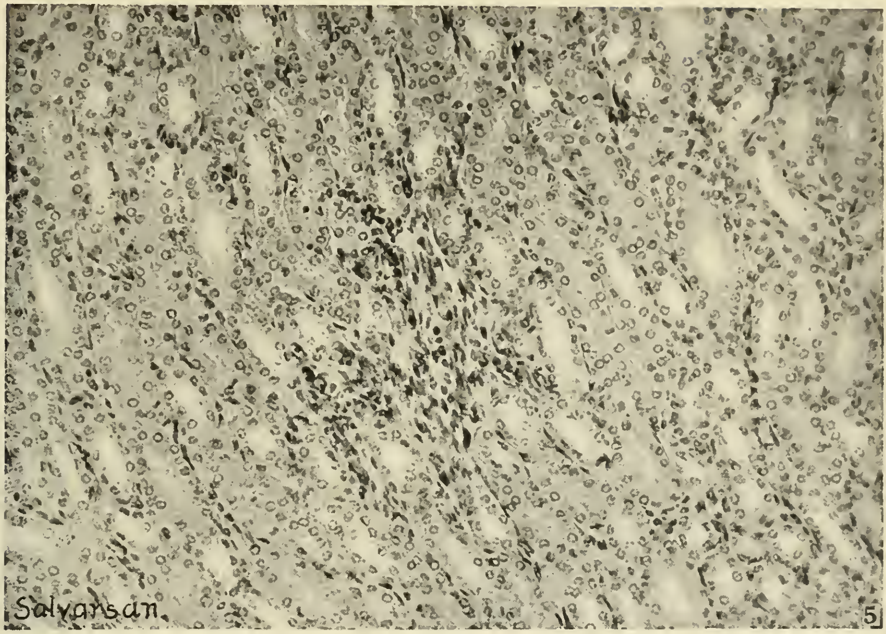
(Pearce and Brown: Renal Injury and Repair.)

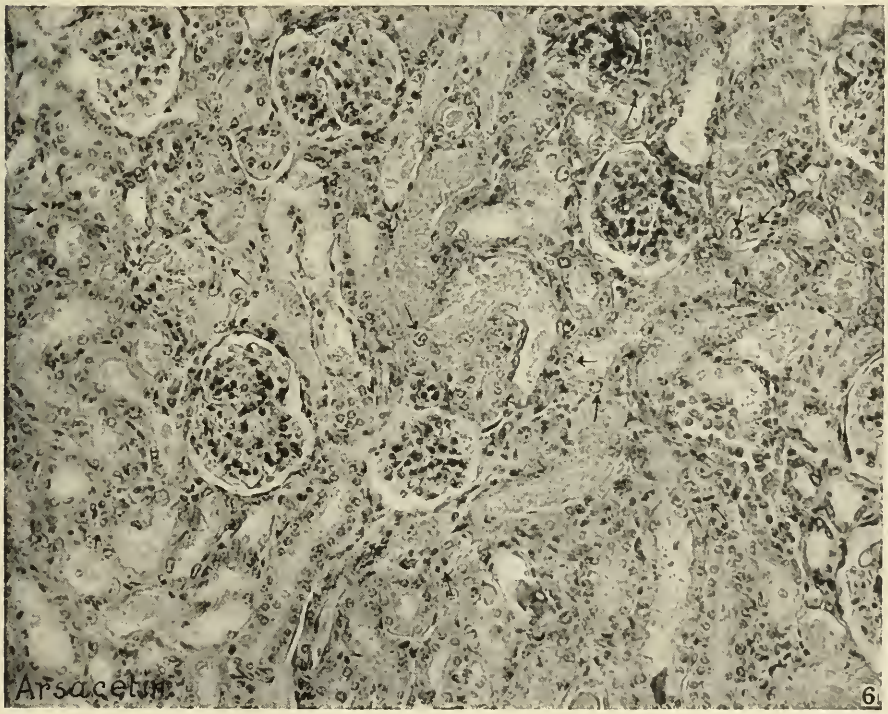




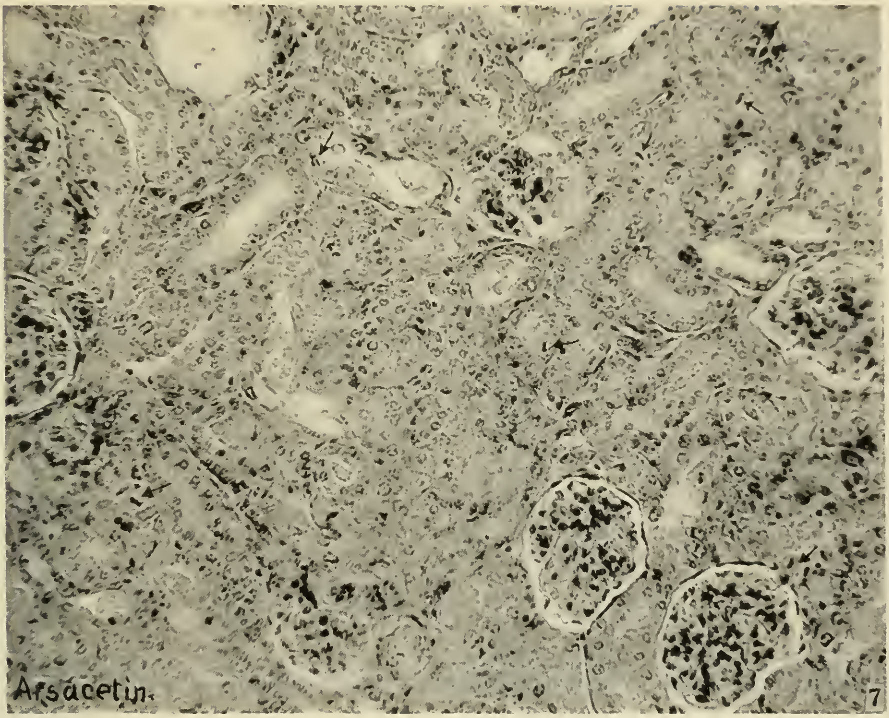
(Pearce and Brown: Renal Injury and Repair.)

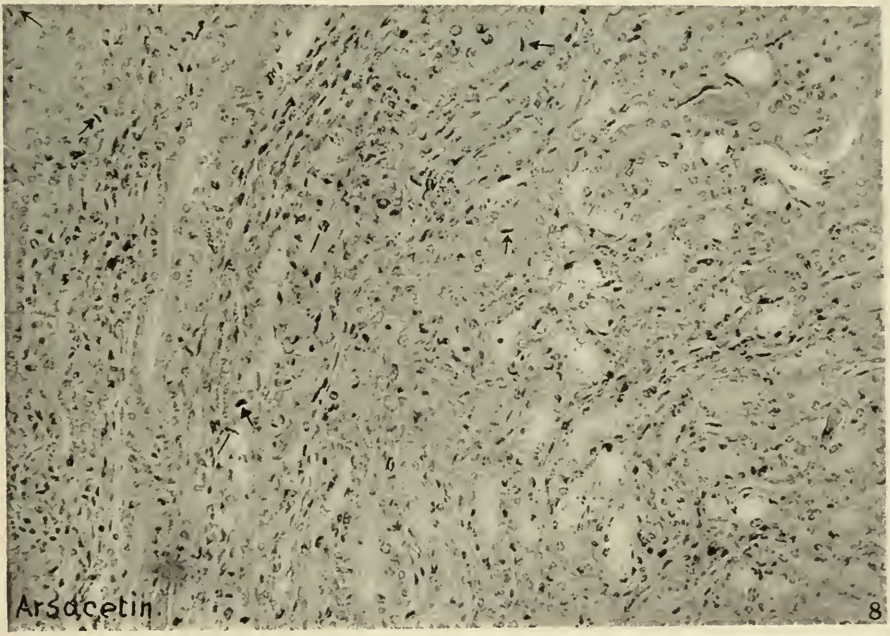
45-83



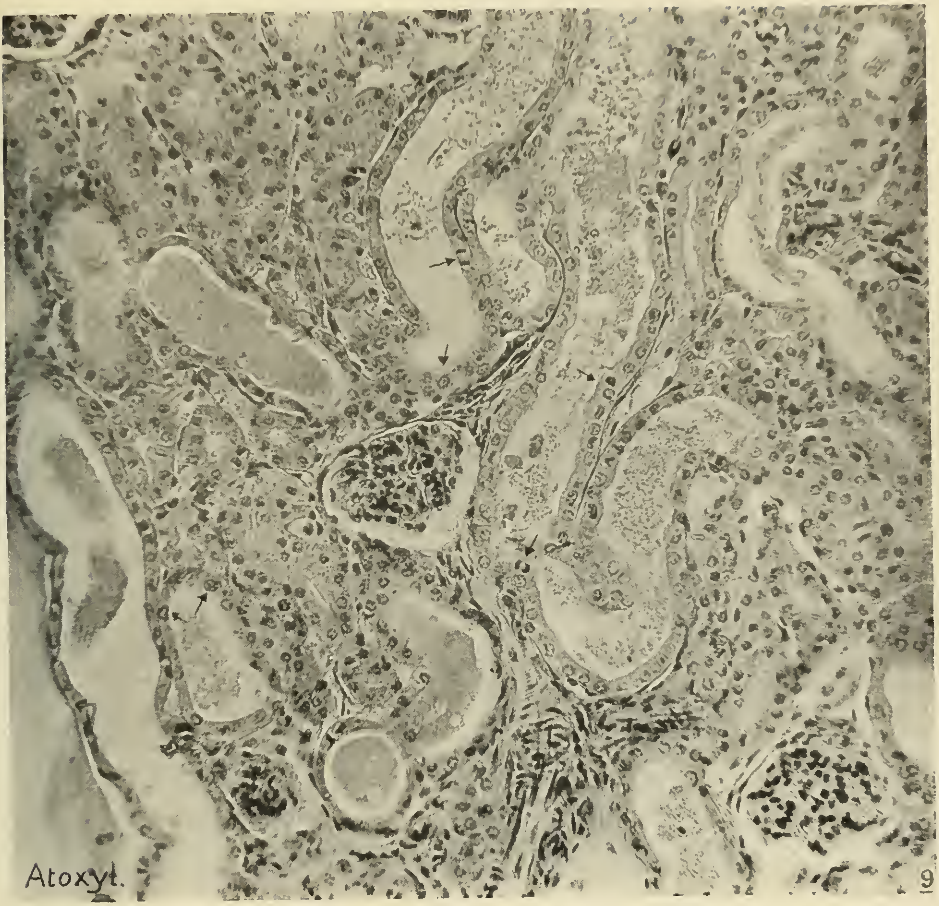


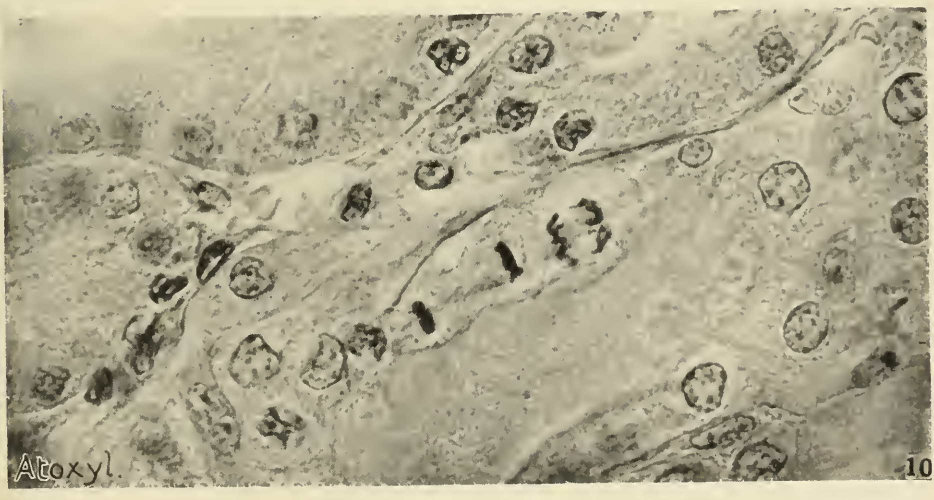
(Pearce and Brown. Renal Injury and Repair.)





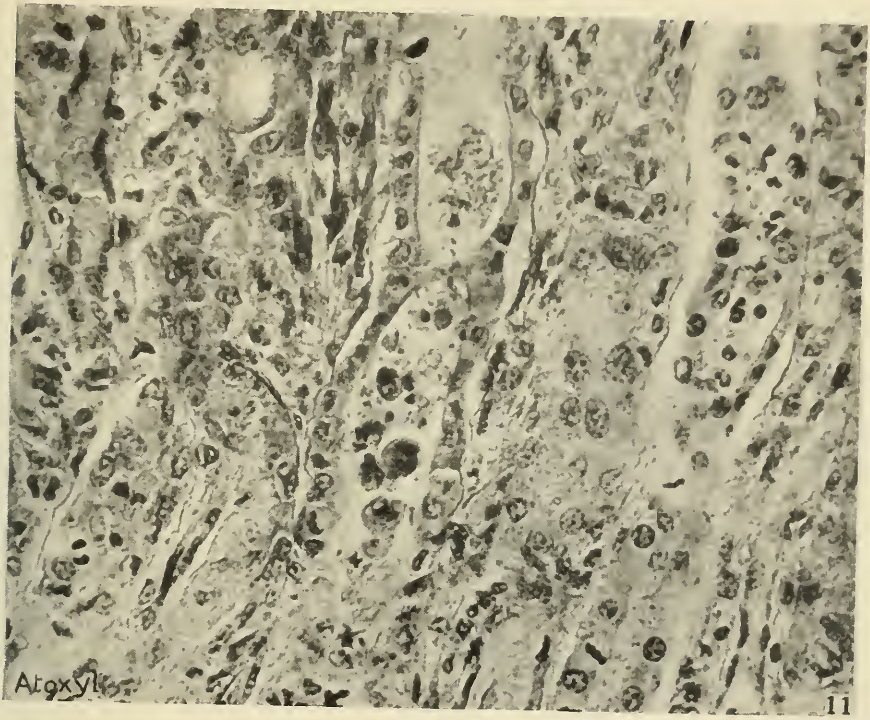
(Pearce and Brown: Renal Injury and Repair.)

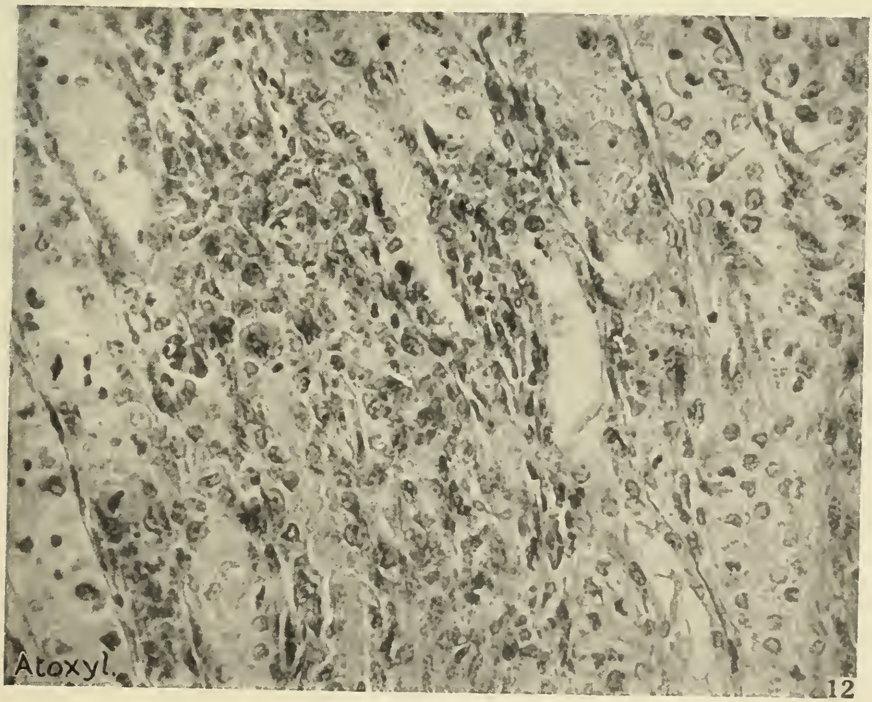




(Pearce: and Brown: Renal Injury and Repair.)

41-86





(Pearce and Brown: Renal Injury and Repair.)

FIG. 1

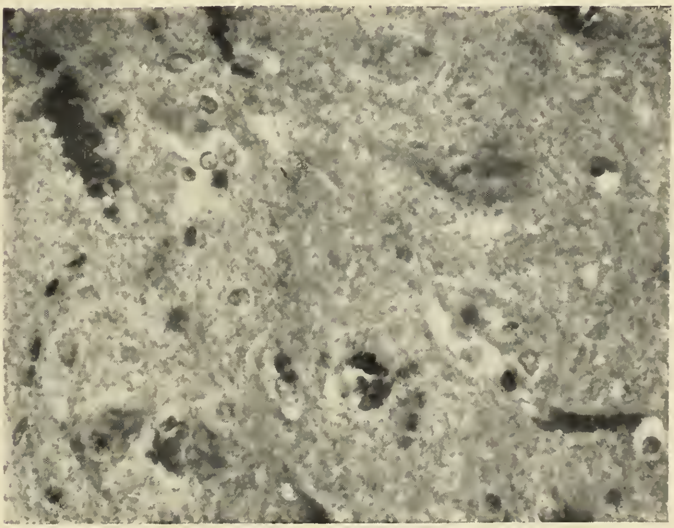


FIG. 2.

(Rosenau and Havens: Poliomyelitis in the Rabbit.)

974

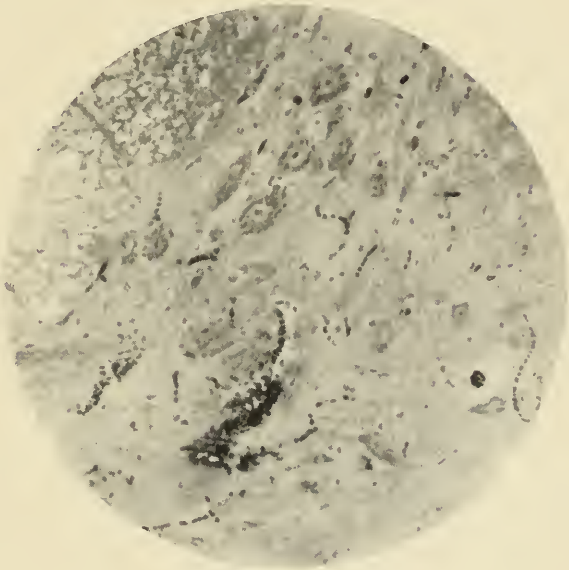


Fig. 3.

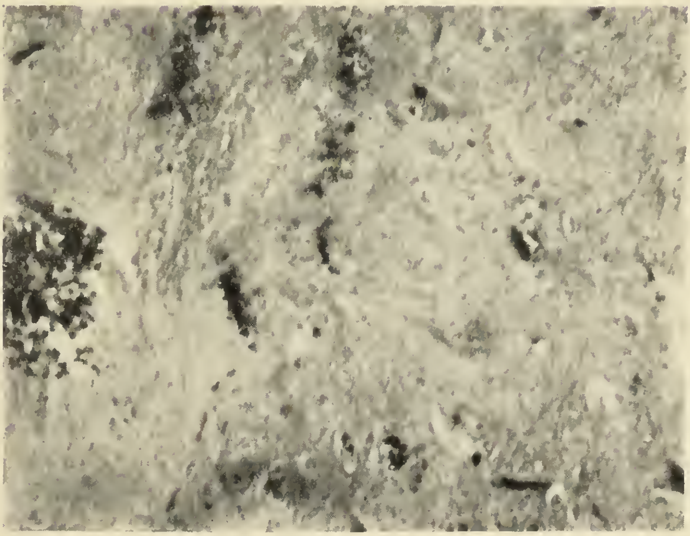


Fig. 4.

(Rosenau and Havens: Poliomyelitis in the Rabbit.)

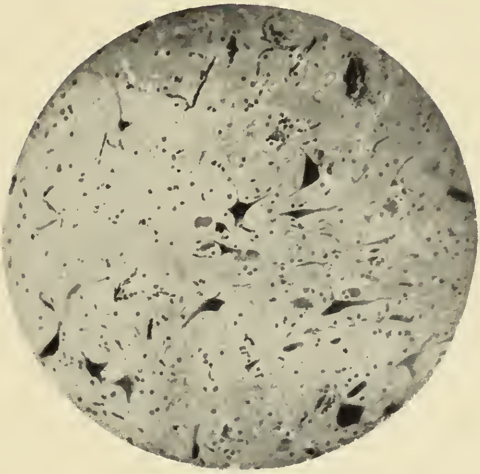


FIG. 5

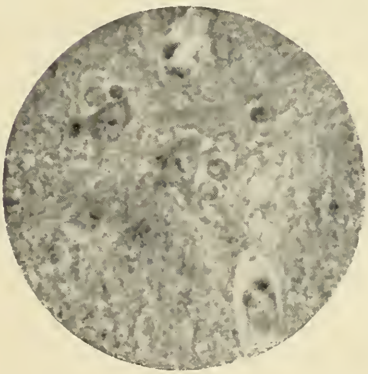


FIG. 6.

(Rosenau and Havens: Poliomyelitis in the Rabbit.)

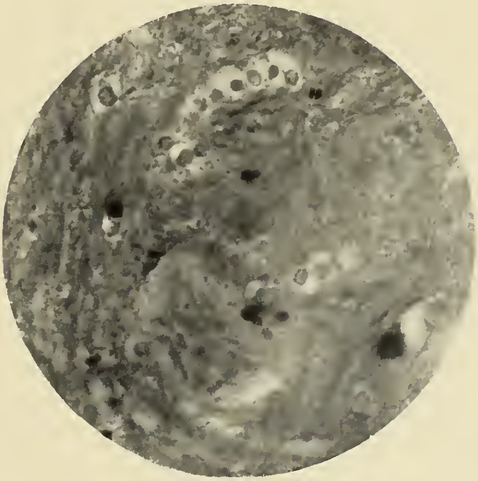


Fig 7.

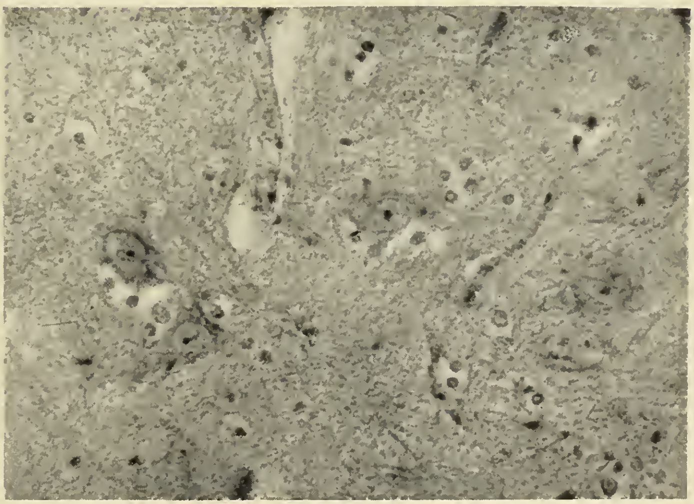


Fig. 8.

(Rosenau and Havens: Poliomyelitis in the Rabbit.)

5481

William P. Didurch
1915

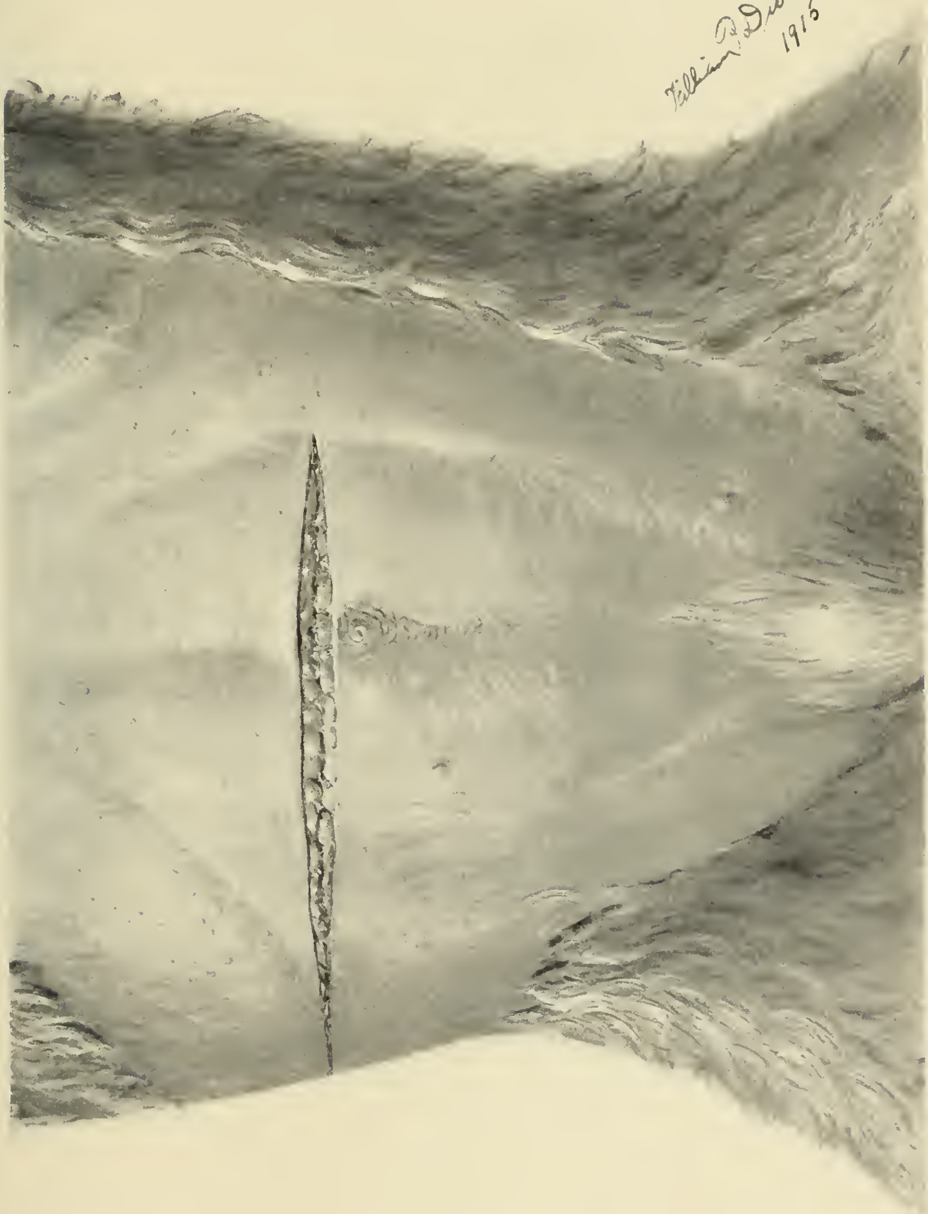


Fig. 1.

(Quinby: Kidney Deprived of Nerves.)

FIG. 2.

(Quinby: Kidney Deprived of Nerves.)

548[3]

FIG. 3.

(Quinby: Kidney Deprived of Nerves.)

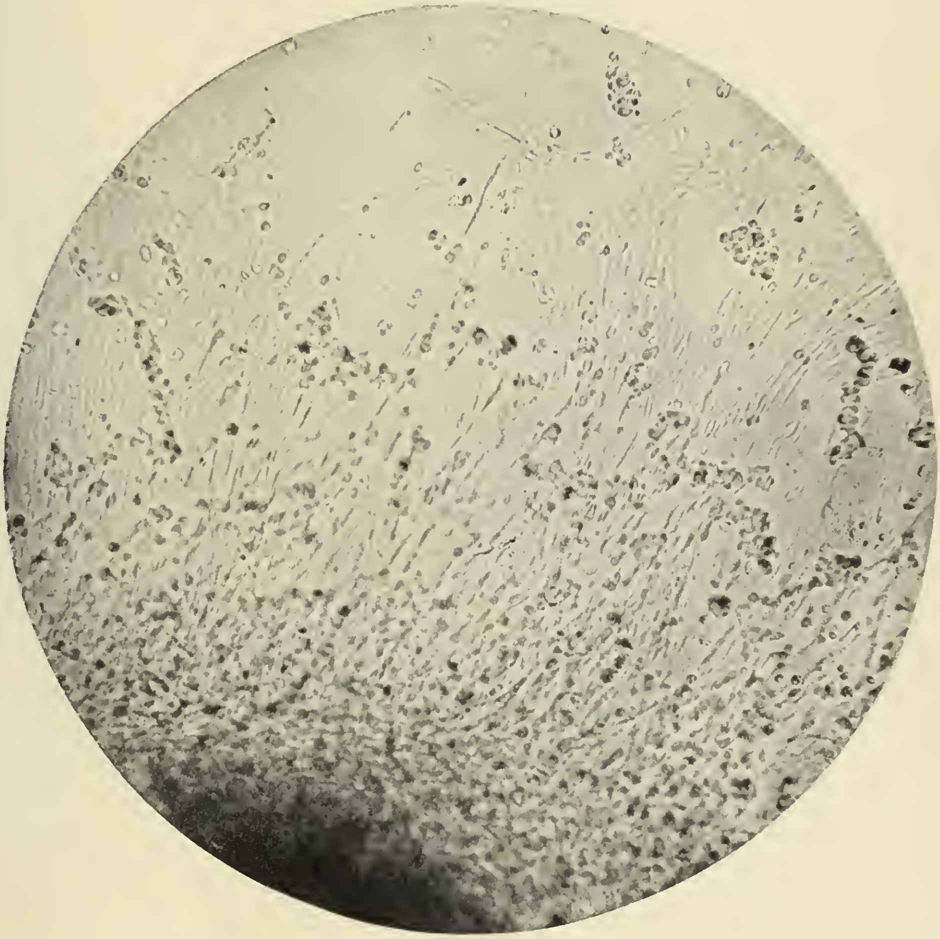
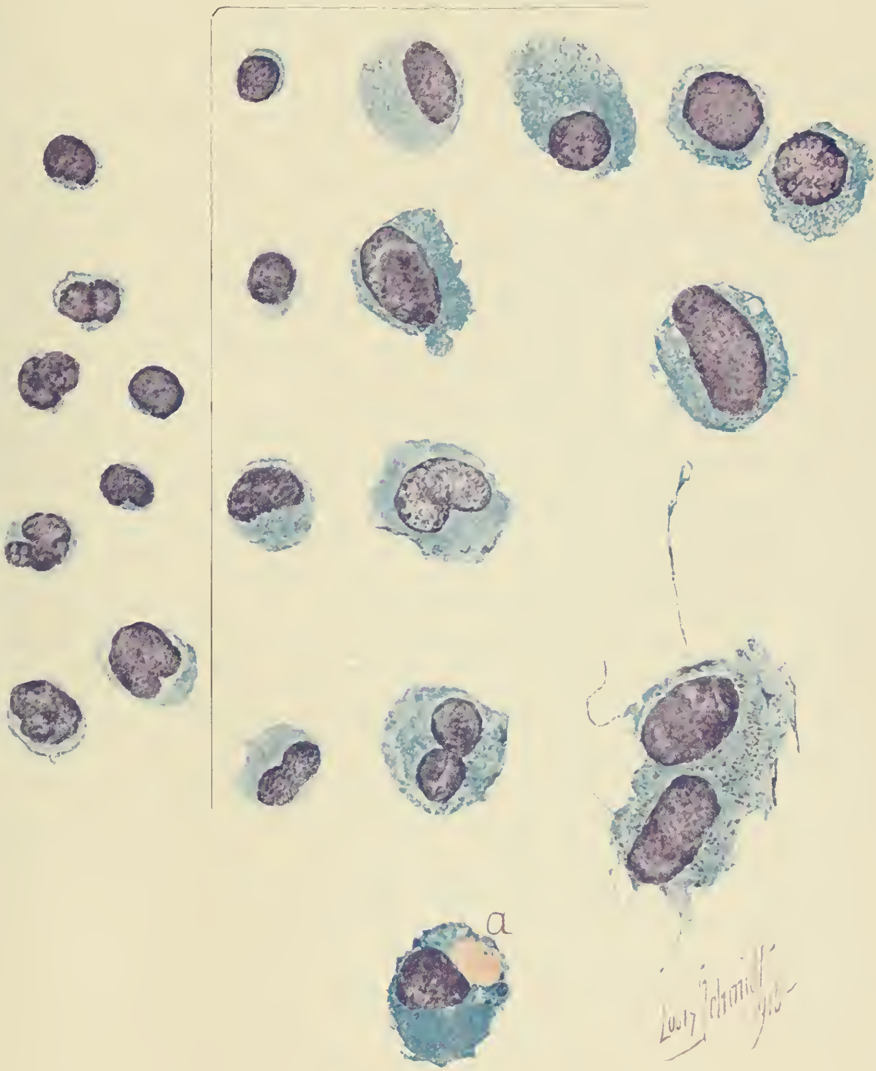


Fig. 1.

(Rous and Jones: Living Cells from Fixed Tissues.)

Blood	Heart		Muscle
Mononuclears	Small Series	Large Series	Large Series



(Rous and Jones: Living Cells from Fixed Tissues.)

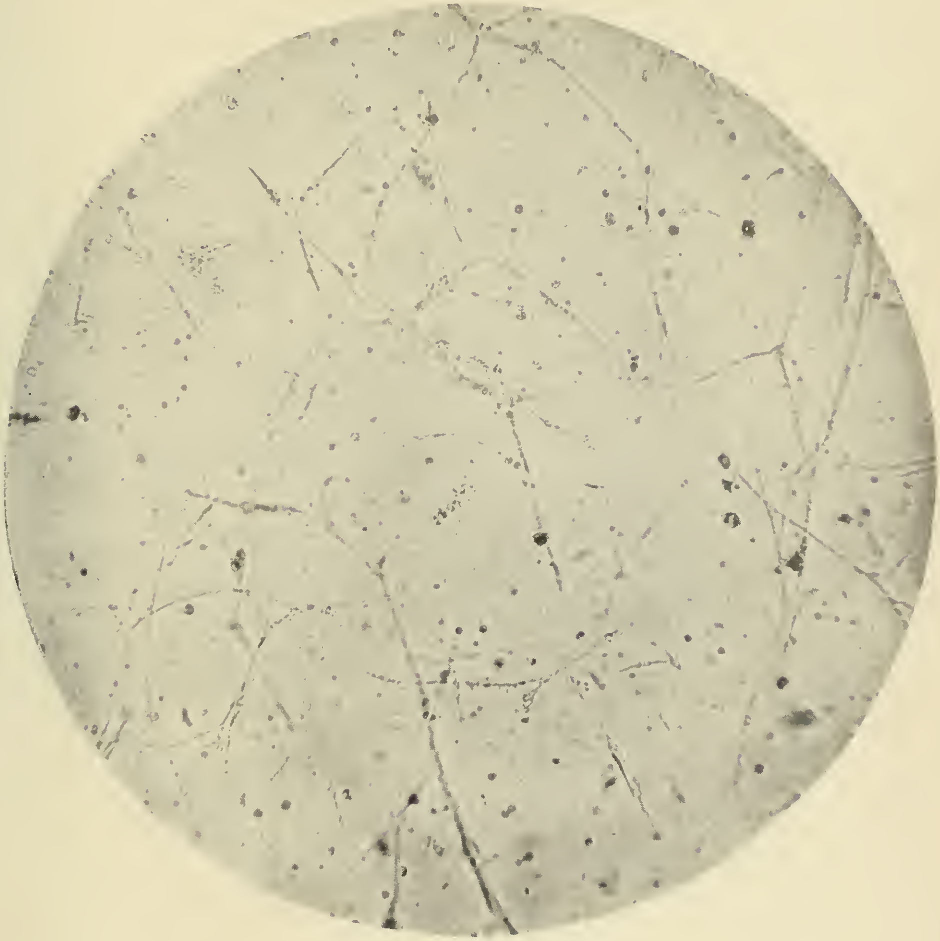


FIG. 3

(Rous and Jones: Living Cells from Fixed Tissues.)

FIG. 4.

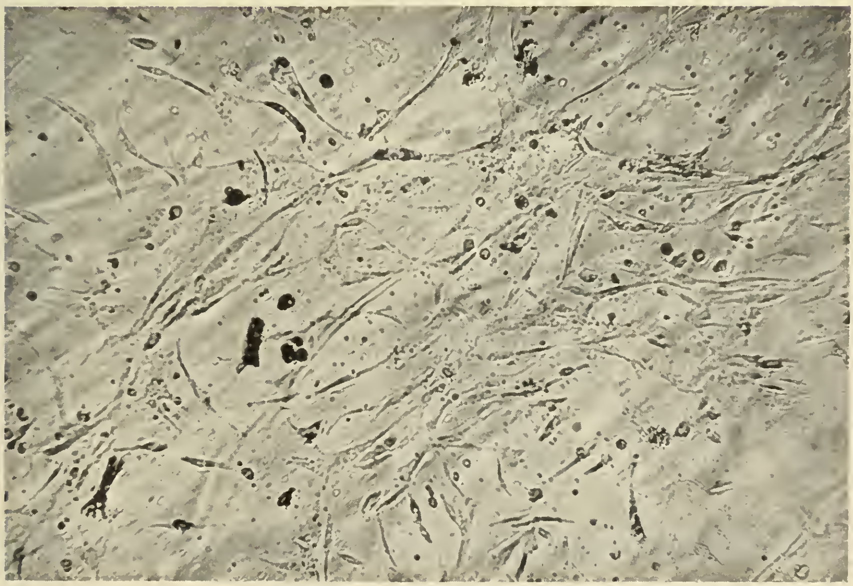


FIG. 5.

(Rous and Jones: Living Cells from Fixed Tissues.)

5 62

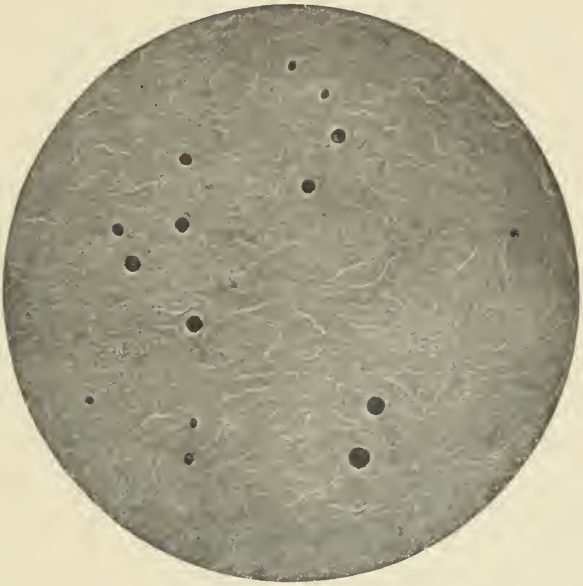


Fig. 1.

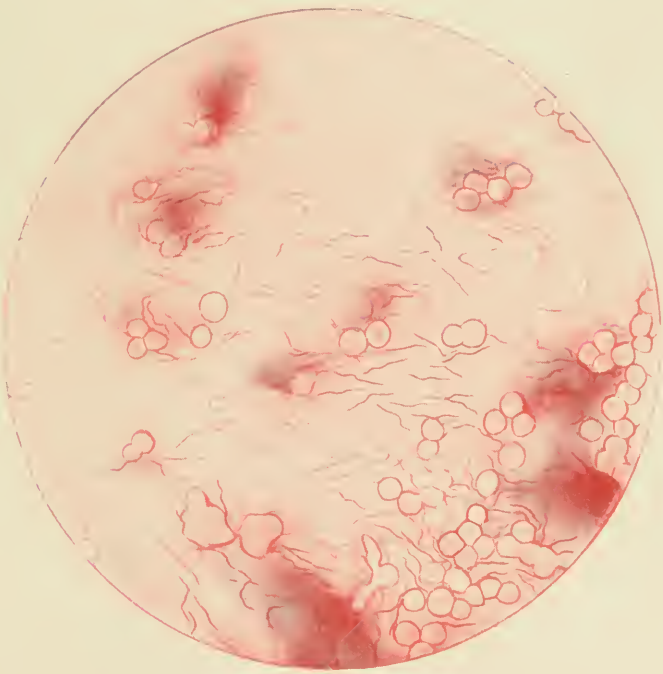
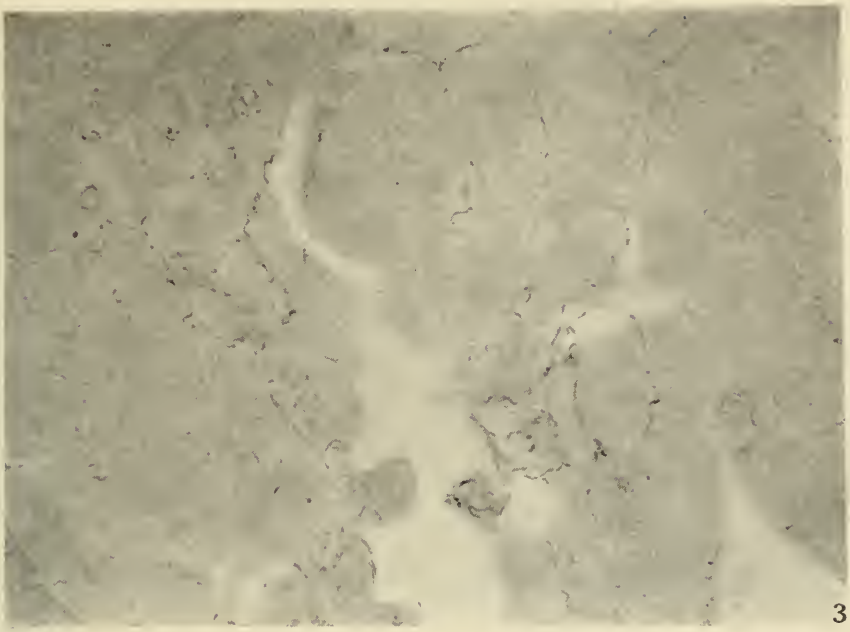
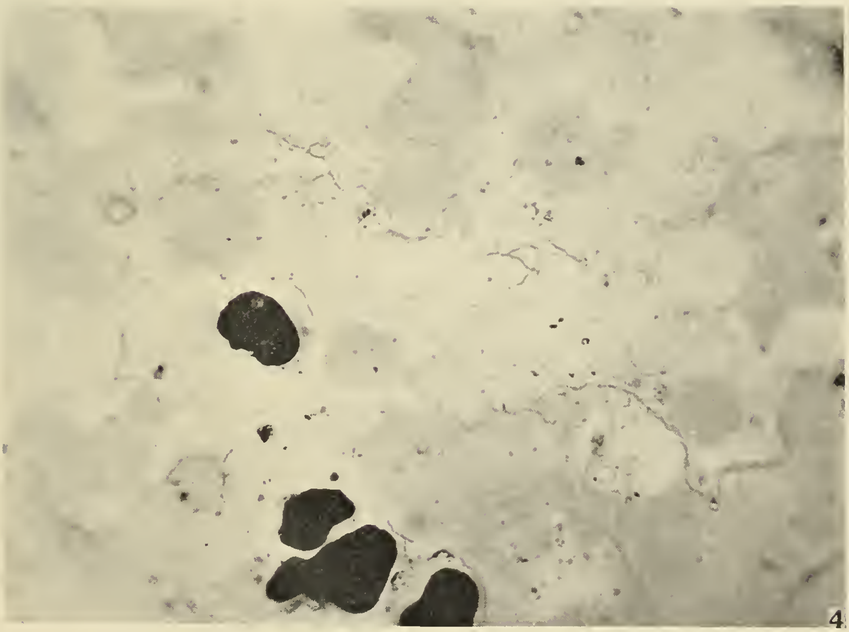


Fig. 2.

(Ito and Matsuzaki: Cultivation of *Spirochæta icterohæmorrhagiæ*.)

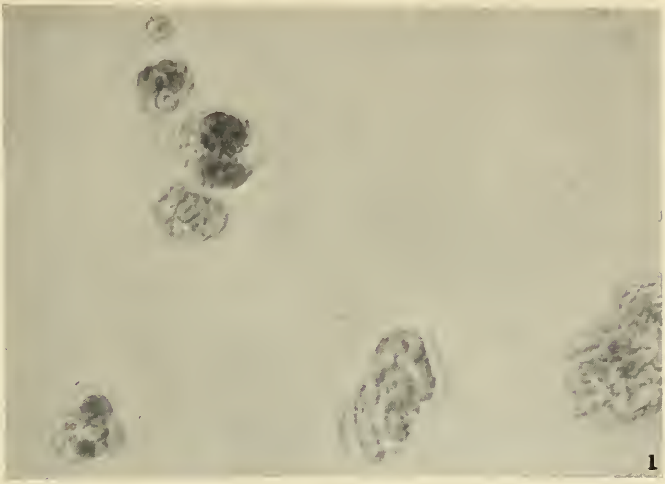


3

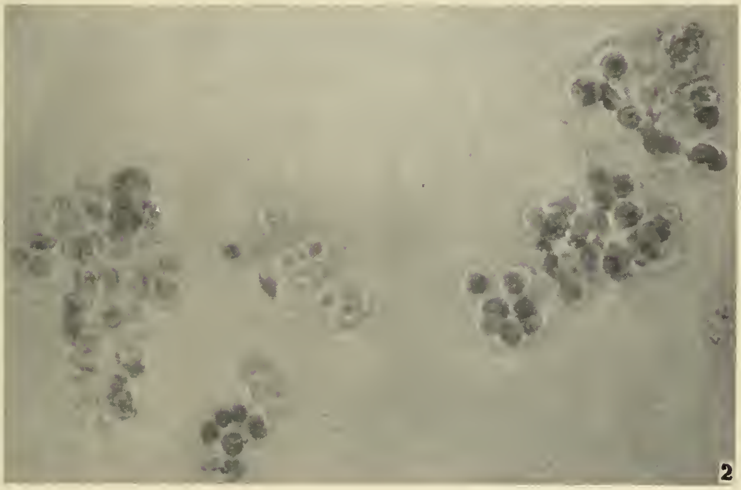


4

(Ito and Matsuzaki: Cultivation of *Spirochæta icterohæmorrhagiæ*.)



1



2

3

(Rous and Jones: Protection of Pathogenic Microorganisms.)

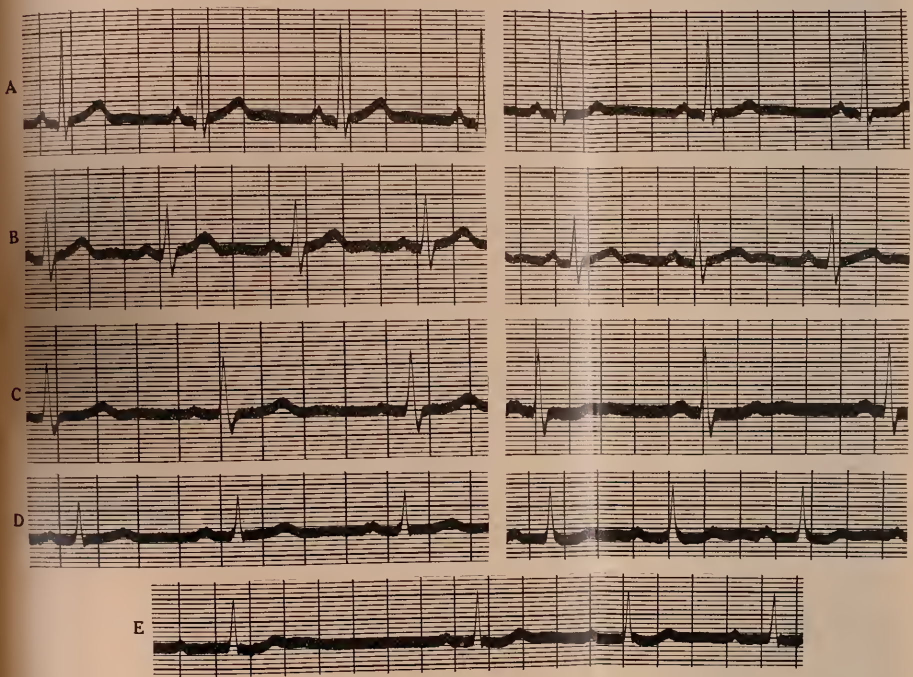


FIG. 1.

(White and Sattler: Effect of Digitalis on Human Electrocardiogram.)

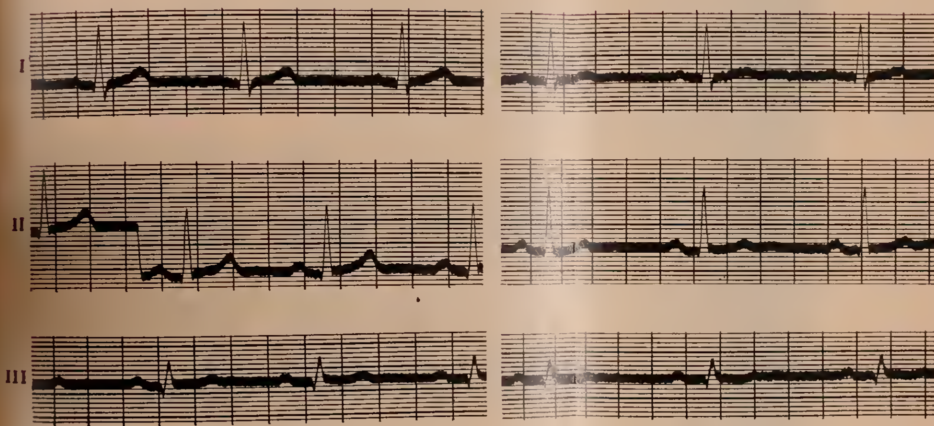


Fig. 2.

(White and Sattler: Effect of Digitalis on Human Electrocardiogram.)

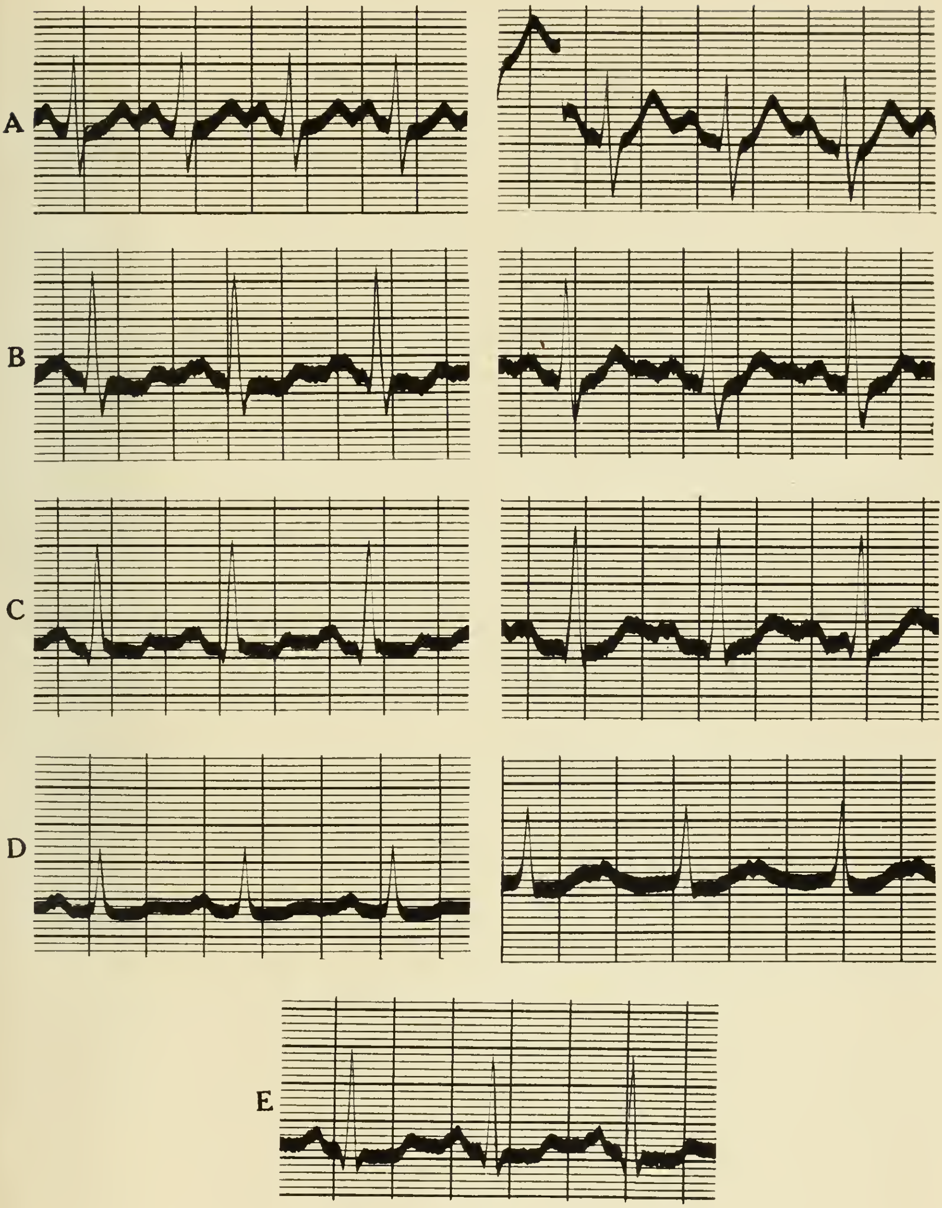


FIG. 3.

(White and Sattler: Effect of Digitalis on Human Electrocardiogram.)

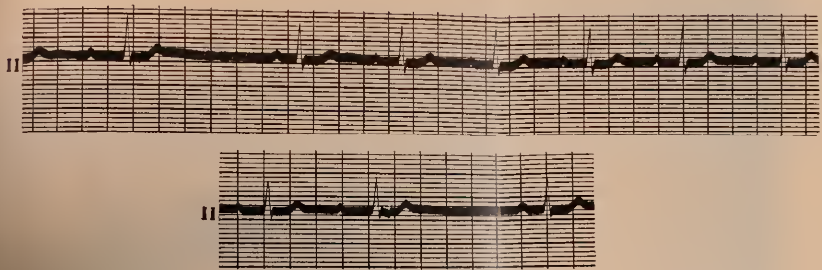


FIG. 4.

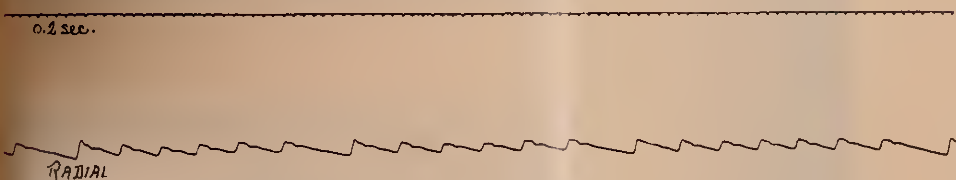


FIG. 5a.

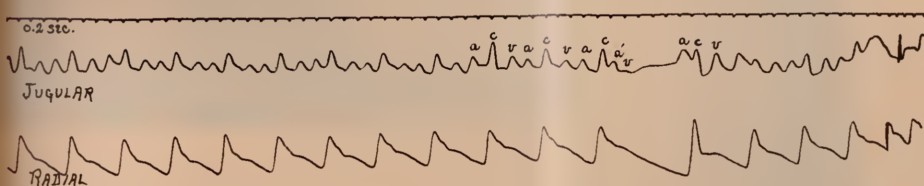


FIG. 5b.

(White and Sattler: Effect of Digitalis on Human Electrocardiogram.)

III II I

FIG. 1.

III II I

FIG. 2.

(Gates and Meltzer: Action of Sodium Oxalate, Magnesium, and Calcium Salts)

FIG. 1.

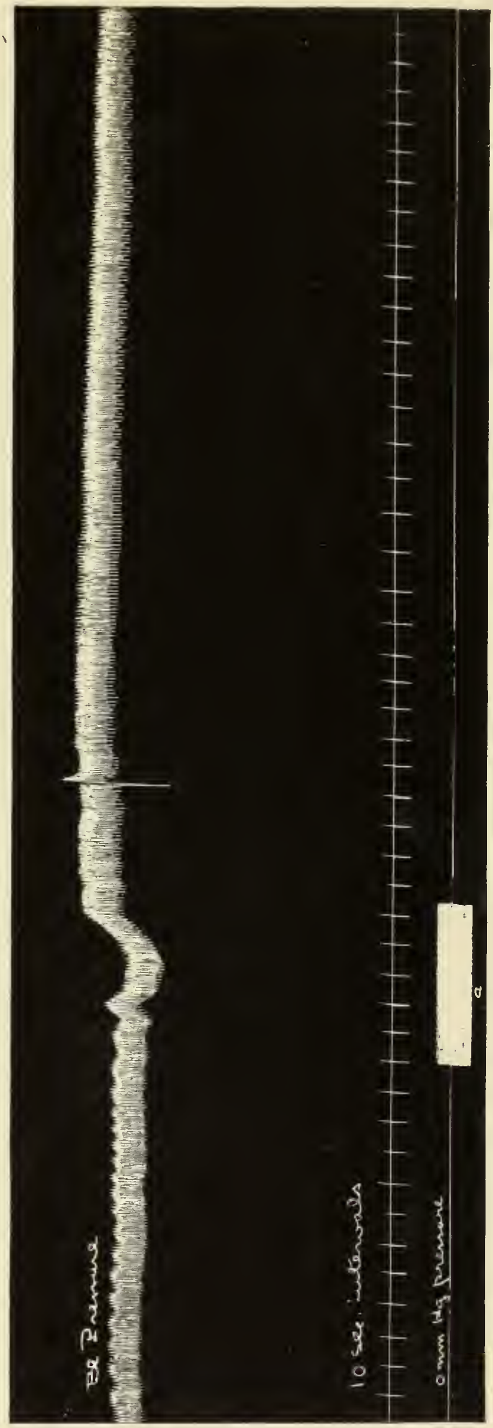


FIG. 2.

(Lewis: Pressor Substance in Fetal Hypophysis.)

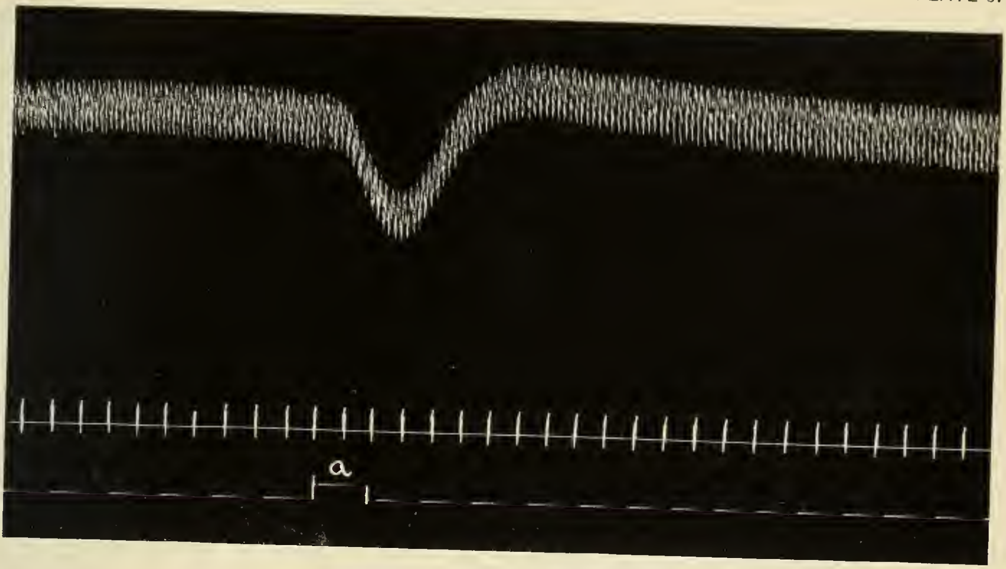


FIG. 3.

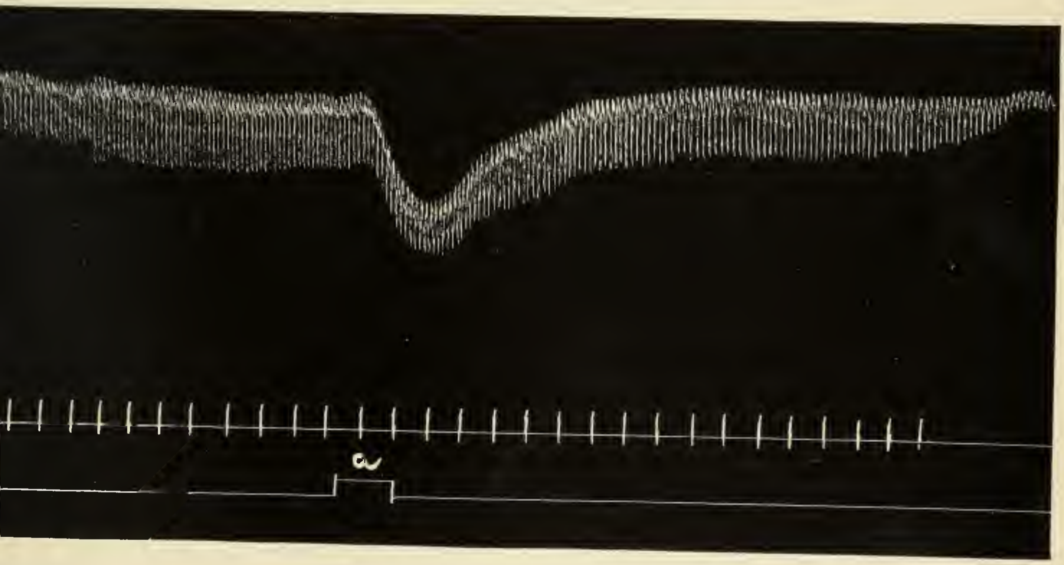


FIG. 4.

(Lewis: Pressor Substance in Fetal Hypophysis.)

716'

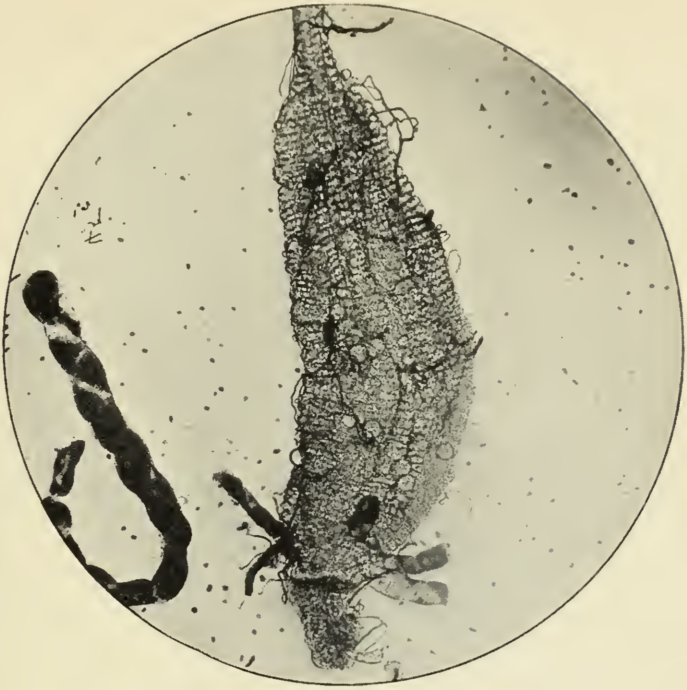


FIG. 1.

FIG. 2.

(King: Development of Malaria Parasites.)

716[2]

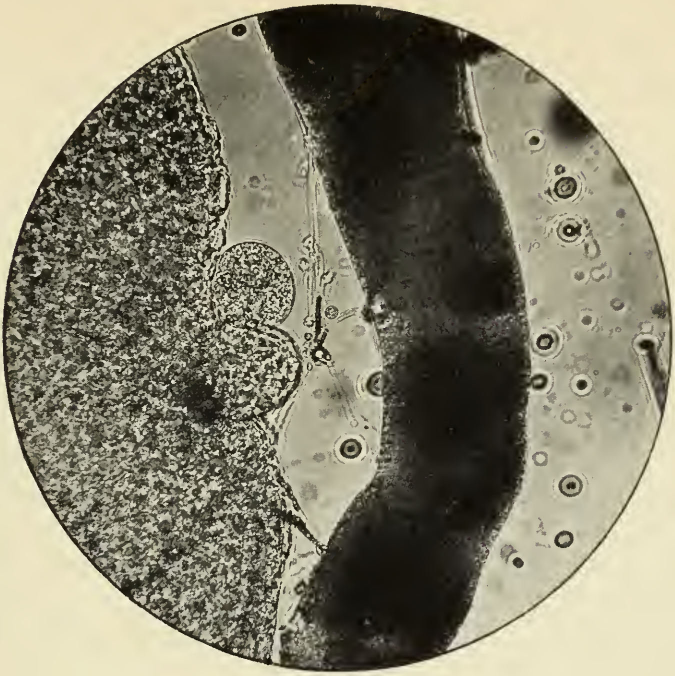


FIG. 3.

FIG. 4.

(King: Development of Malaria Parasites.)

Fig. 5.

Fig. 6.

(King: Development of Malaria Parasites.)

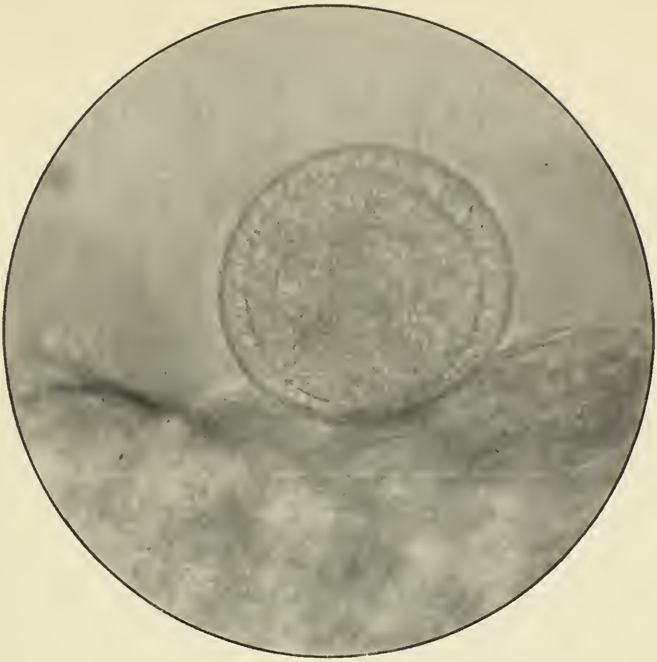


FIG. 7.

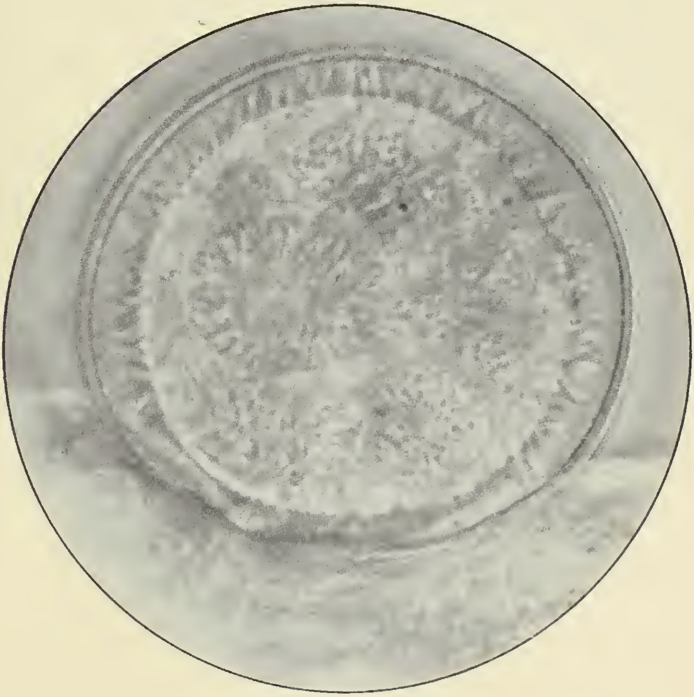


FIG. 8.

(King: Development of Malaria Parasites.)

716

FIG. 9.

FIG. 10.

FIG. 11.

(King: Development of Malaria Parasites.

716

FIG. 12.

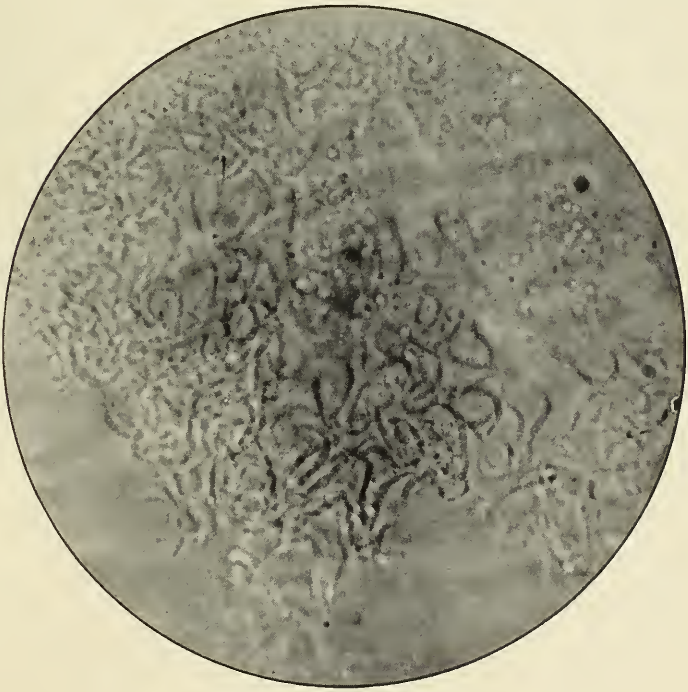


FIG. 13.

(King: Development of Malaria Parasites.)

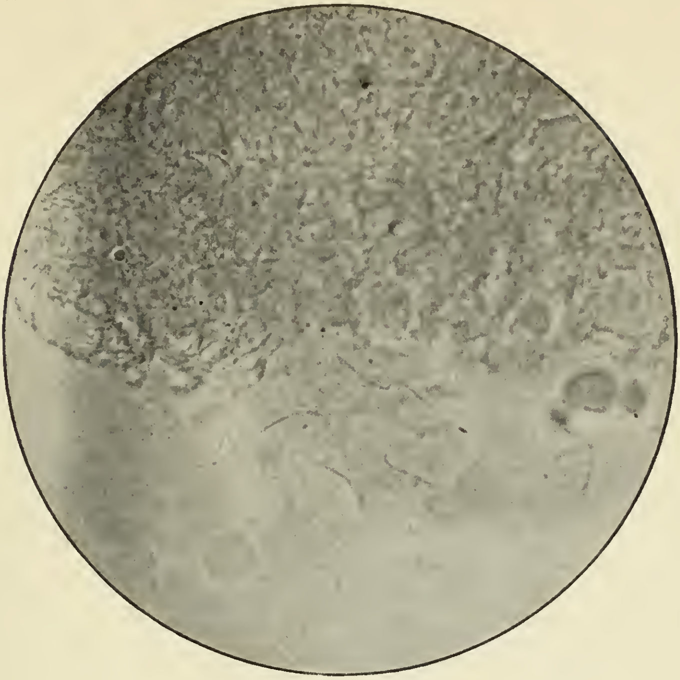


FIG. 14.

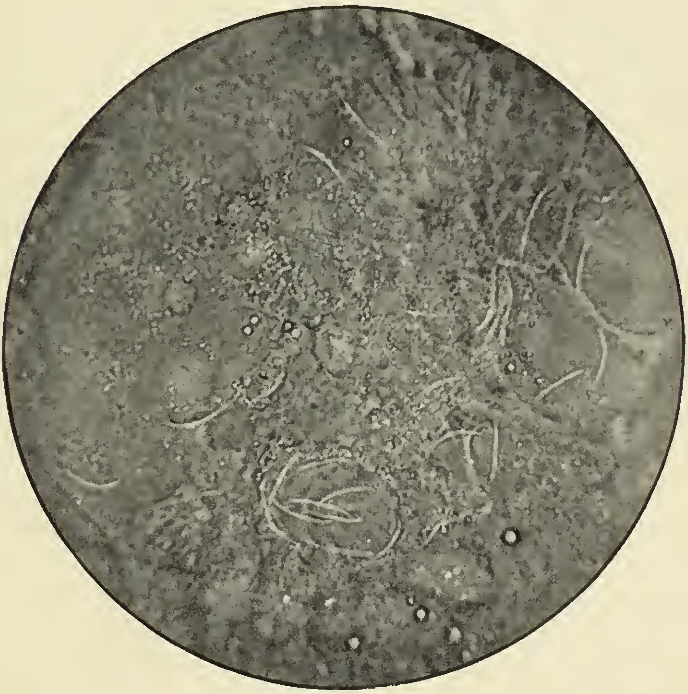


FIG. 15.

(King: Development of Malaria Parasites.)

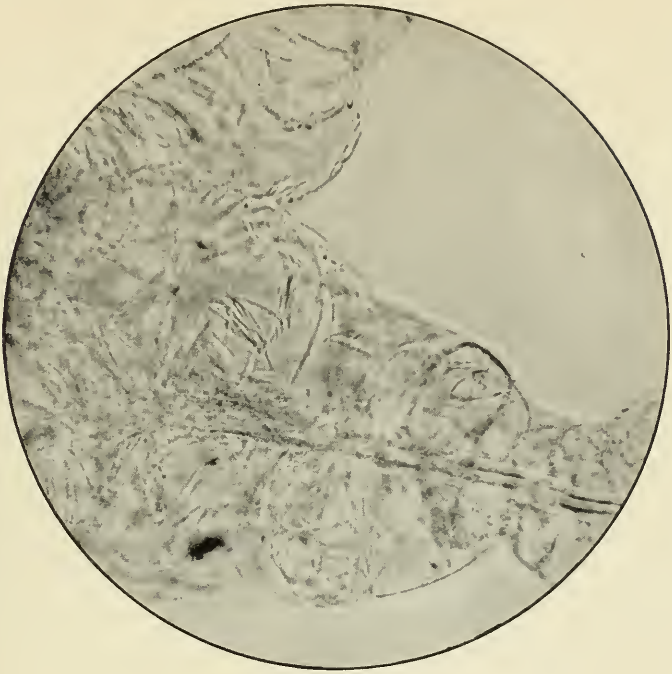


FIG. 16.

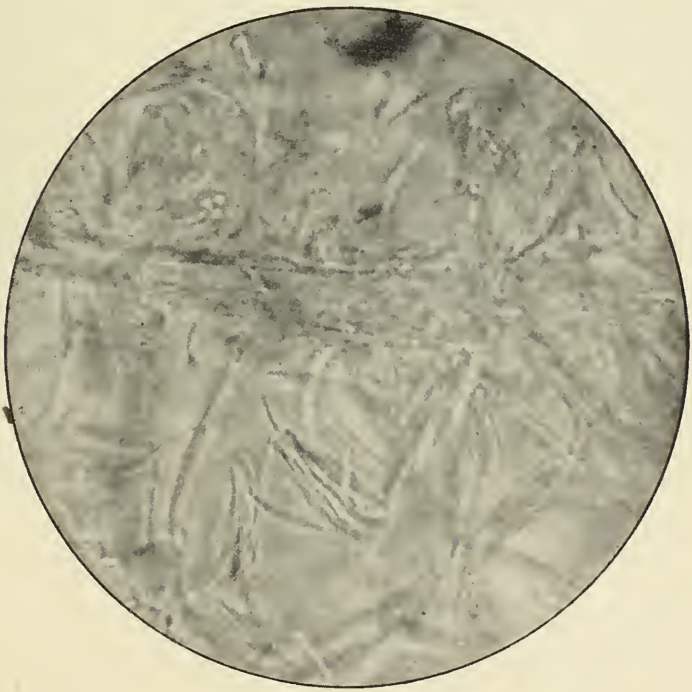


FIG. 17.

(King: Development of Malaria Parasites.)

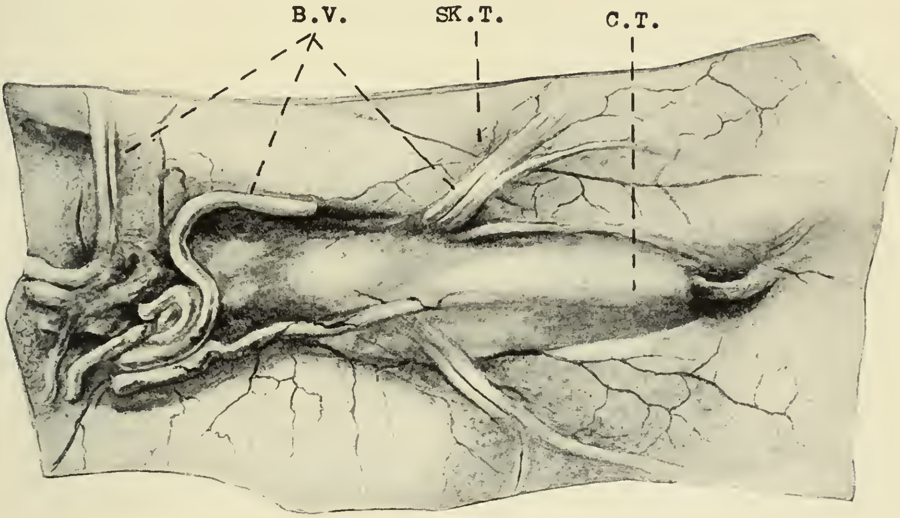


Fig. 1.

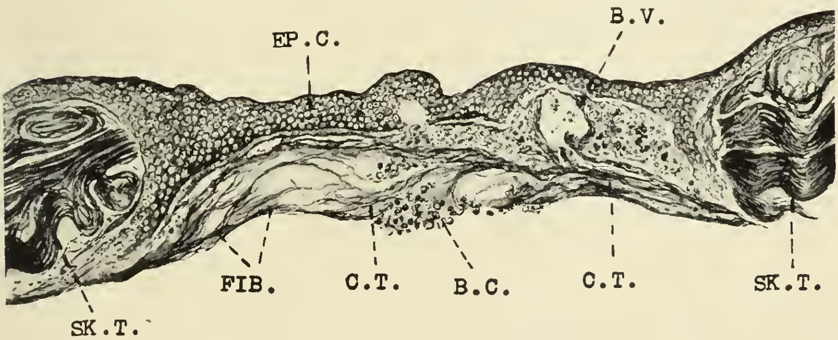


Fig. 2.

(Baitsell: Fibrous Tissue Formed in Wound Healing.)

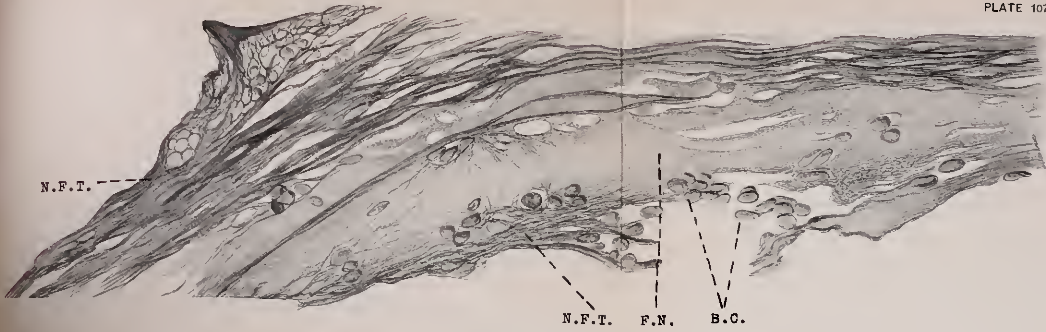


FIG. 3.

FIG. 4.

FIG. 5.

(Baitsell: Fibrous Tissue Formed in Wound Healing.)

7567[3]

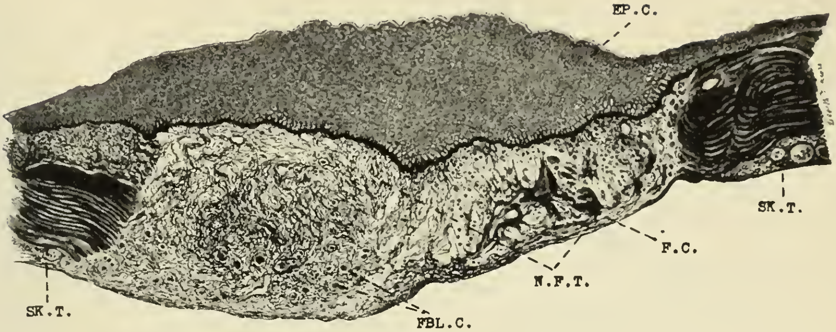


FIG. 6.

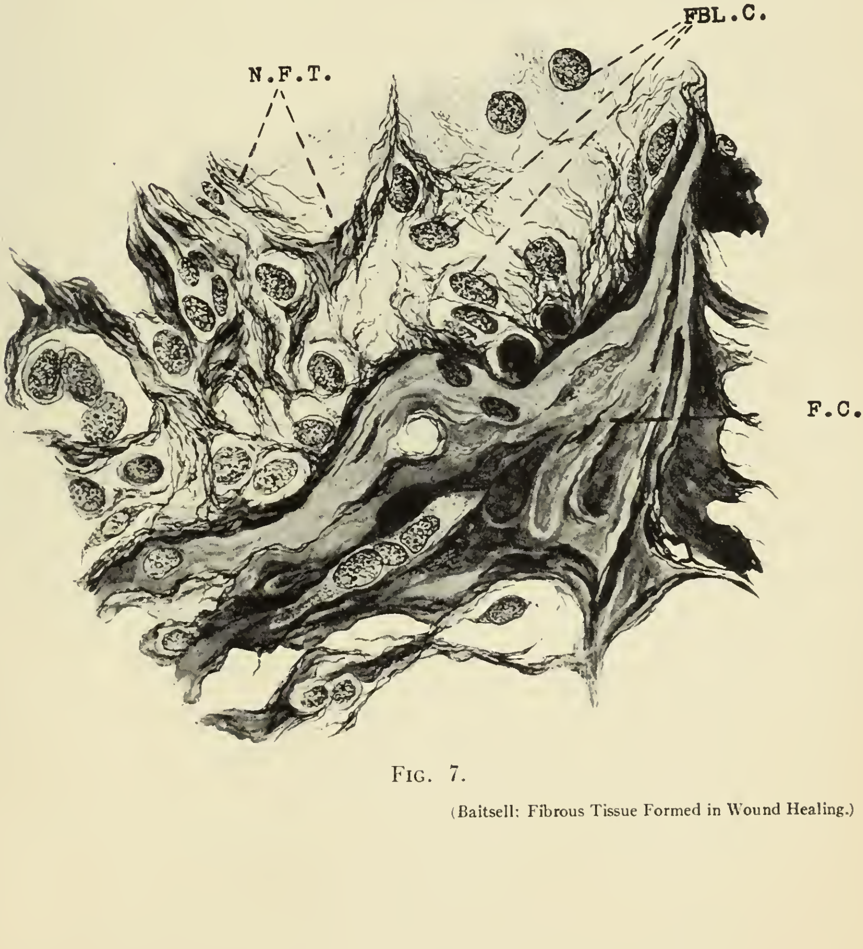


FIG. 7.

(Baitsell: Fibrous Tissue Formed in Wound Healing.)

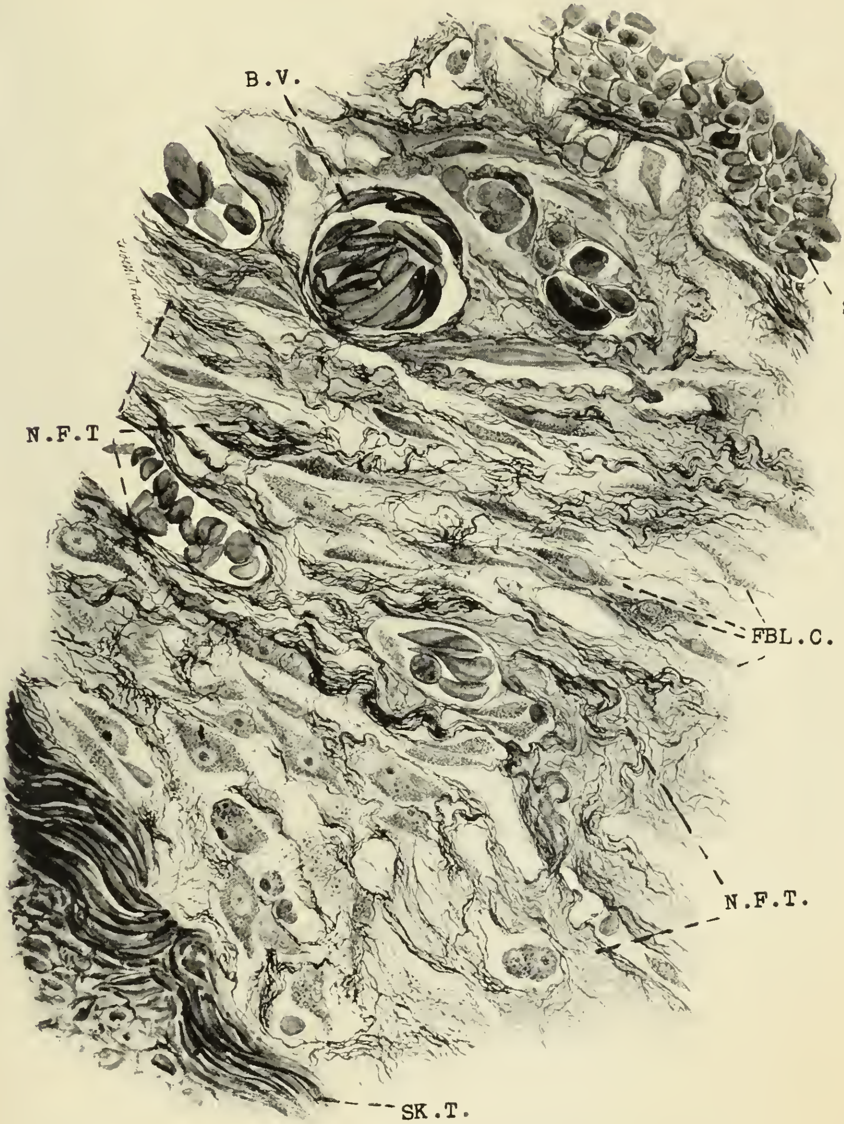


FIG. 8.

(Baitsell: Fibrous Tissue Formed in Wound Healing.)

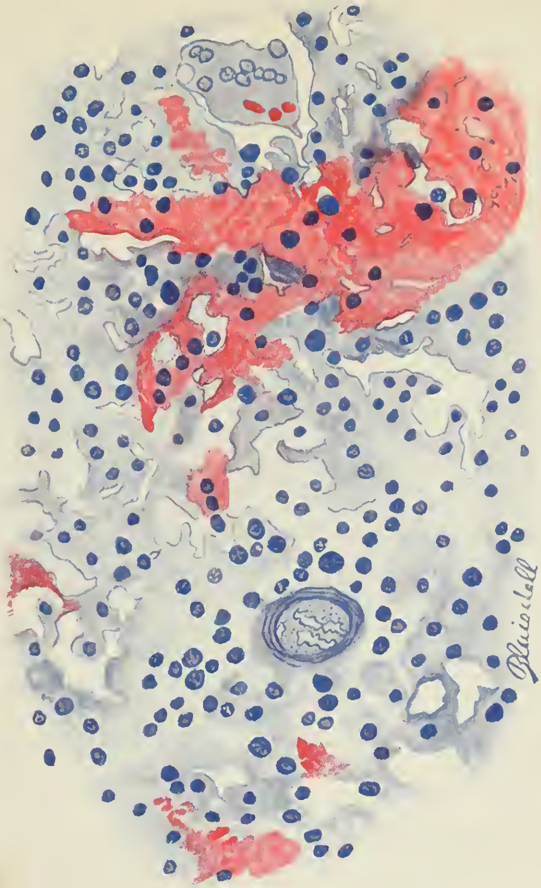


FIG. 1.

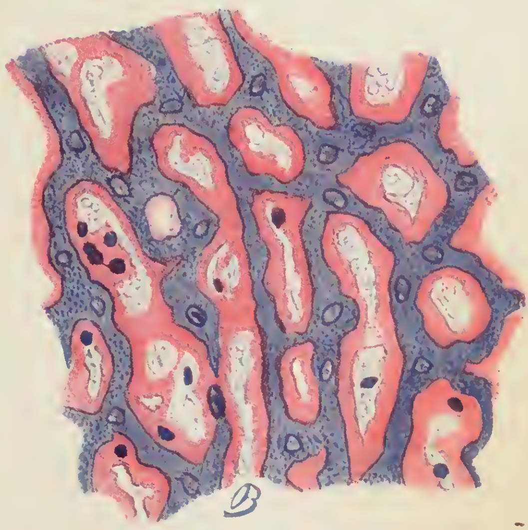


FIG. 2.

(Bailey: Amyloid Disease and Chronic Nephritis.)

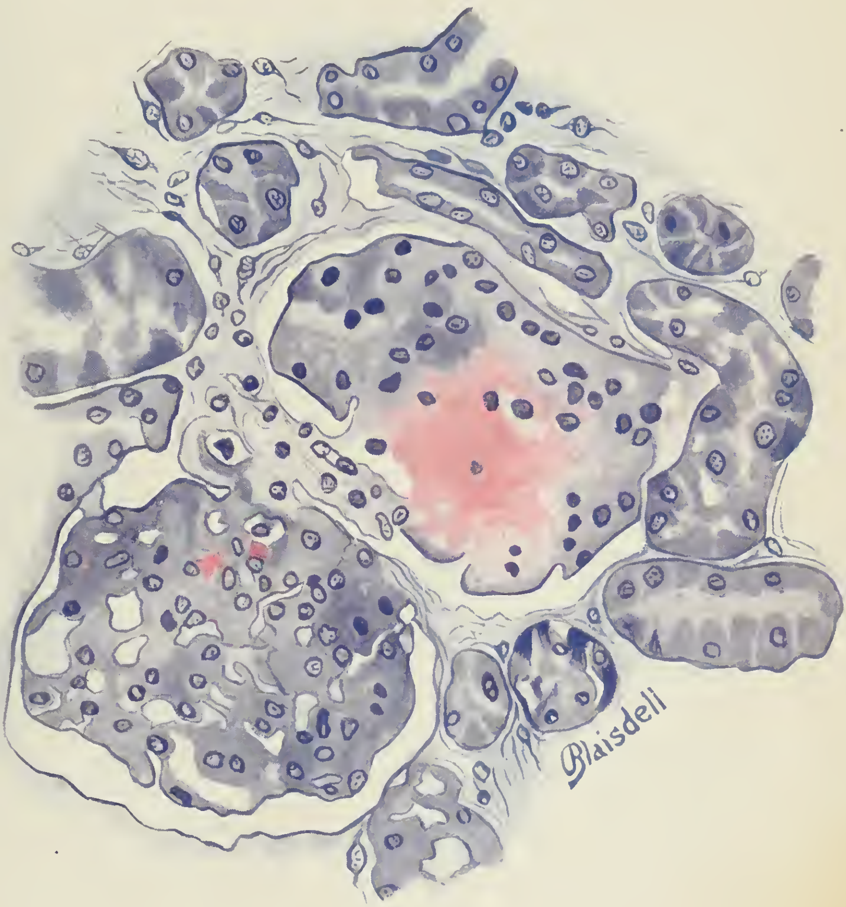


Fig. 3.

(Bailey: Amyloid Disease and Chronic Nephritis.)

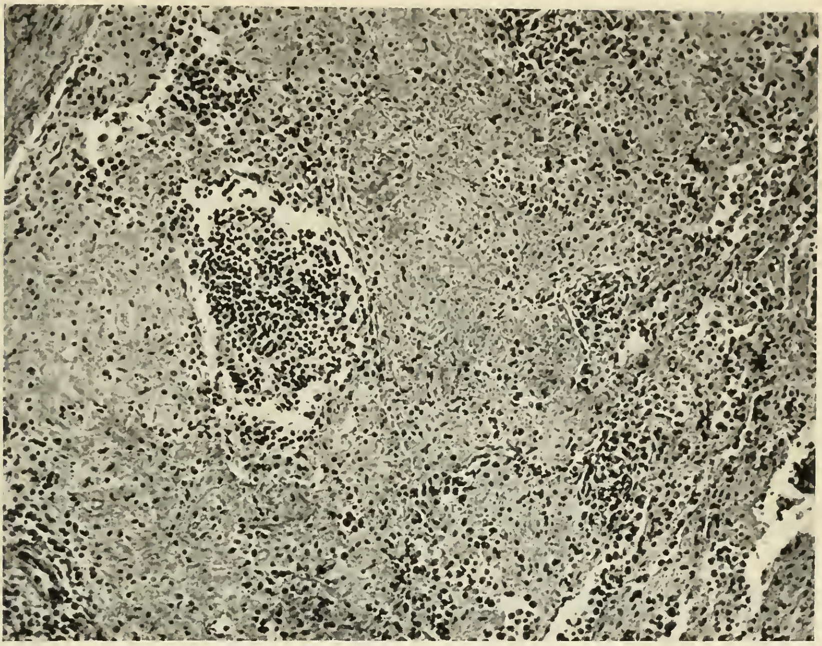


Fig. 4.

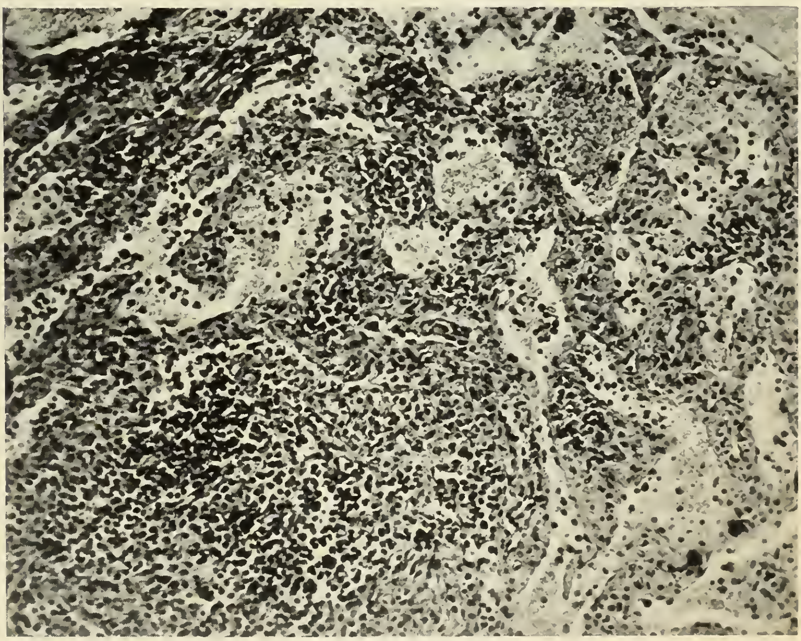


Fig. 5.

(Bailey: Amyloid Disease and Chronic Nephritis.)

FIG. 6.

(Bailey: Amyloid Disease and Chronic Nephritis.)

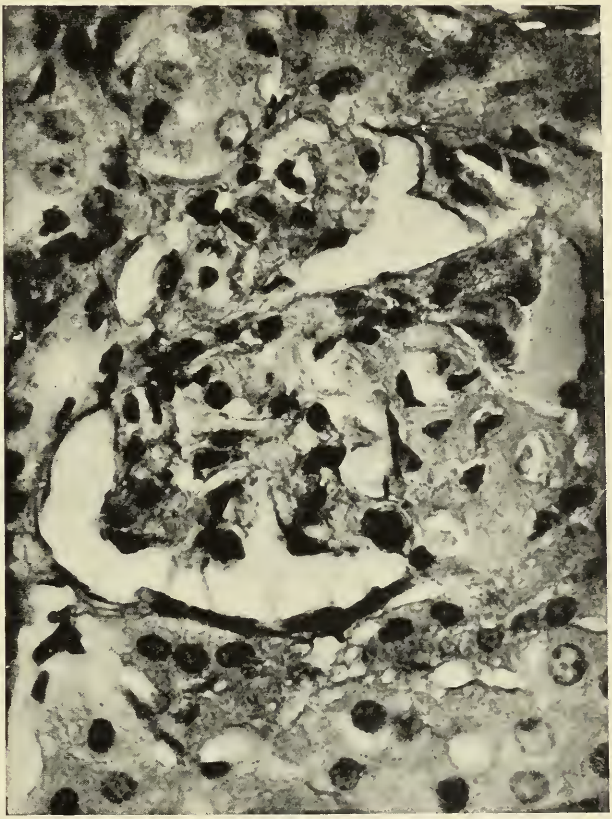


FIG. 7.

(Bailey: Amyloid Disease and Chronic Nephritis.)

790

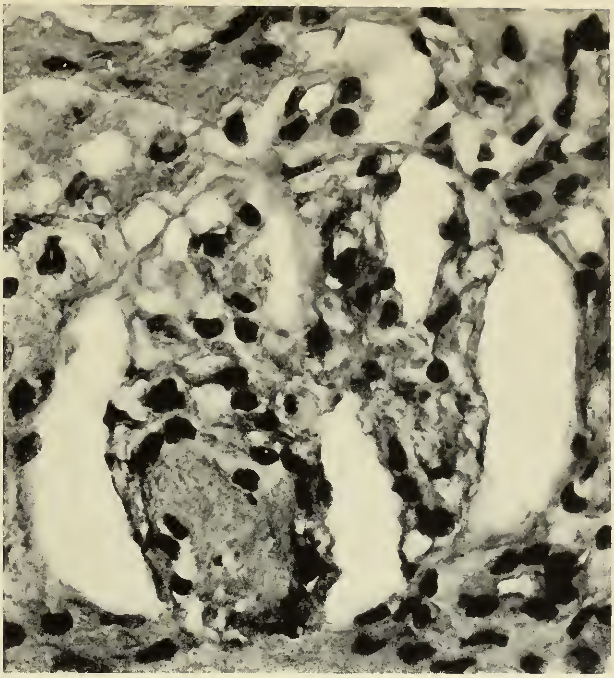


Fig. 8.

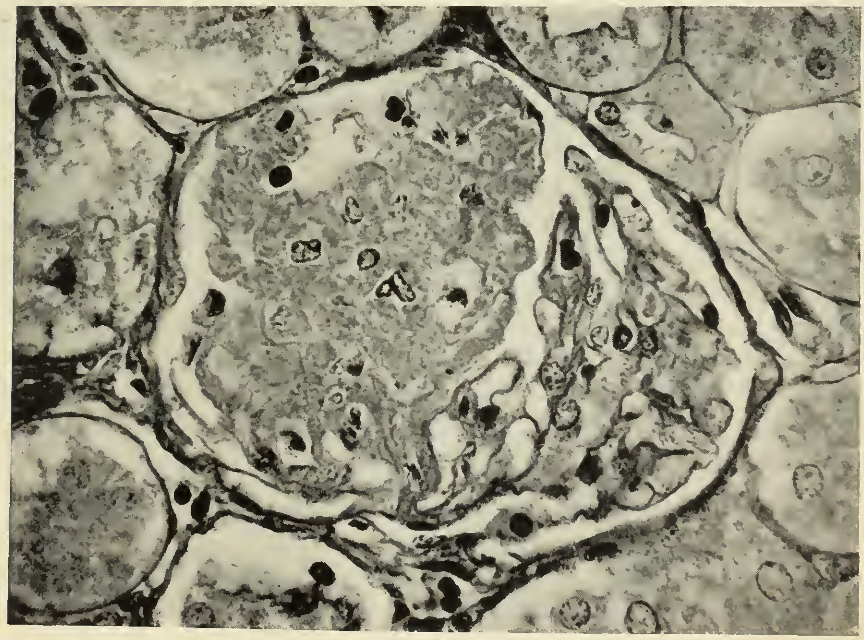


Fig. 9.

(Bailey: Amyloid Disease and Chronic Nephritis.)